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(57) Abstract

Novel transgenic plants that have, stably integrated into their nuclear genome, a maintainer gene comprising a fertility-restorer gene and a pollen-lethality gene. The plants can be used to maintain a homogeneous population of male-sterile plants.

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MAINTENANCE OF MALE-STERILE PLANTS

This invention relates to a process for maintaining male-sterile plant lines that can be used for the production of hybrid seed of a crop, to maintainer plants that can be used in such a process, and to maintainer genes that can be used to produce such maintainer plants.

Background of the Invention

In many, if not most, plant species, the development of hybrid cultivars is highly desired because of their generally increased productivity due to heterosis: the superior performance of hybrid individuals compared with their parents (see, e.g., Fehr (1987) "Principles of Cultivar Development, Volume 1: Theory and Technique", MacMillan Publishing Company, New York; Allard (1960) "Principles of Plant Breeding", John Wiley and Sons, Inc., New York).

The development of hybrid cultivars of various plant species depends upon the capability to achieve almost complete cross-pollination between parents. This is most simply achieved by rendering one of the parent lines male-sterile (i.e., with pollen being absent or nonfunctional), for example, by manually removing the one parent's anthers or by providing the one parent with naturally occurring cytoplasmic or nuclear genes that prevent anther and/or pollen development and/or function, using classical breeding techniques (for a review of the genetics of male-sterility in plants, see Kaul (1988) "Male Sterility in Higher Plants", Springer Verlag, New York).

For hybrid plants where the seed is the harvested product (e.g., corn and oilseed rape), it is, in most cases, also necessary to ensure that fertility of the hybrid plants is fully restored. In plants in which the male-sterility is under genetic control, this requires the use of genes that can restore male-fertility. Hence, the development of hybrid cultivars is mainly dependent on the availability of suitable and effective sterility and restorer genes.

Endogenous nuclear loci are known for most plant species that contain genotypes which effect male-sterility, and generally, such loci need to be homozygous for particular recessive alleles in order to result in a male-sterile phenotype. The presence of a dominant male-fertile allele at such loci results in male-fertility.

Recently, it has been shown that male-sterility can be induced in a plant by providing the plant with a nuclear male-sterility genotype that includes a chimaeric male-sterility gene comprising a DNA sequence (or male-sterility DNA) coding, for example, for a cytotoxic product (such as an RNase) and under the control of a promoter which is predominantly active in selected tissue of the plant's male reproductive organs. In this regard, tapetum-specific promoters, such as the promoter of the TA29 gene of Nicotiana tabacum, have been shown to be particularly useful for this purpose (Mariani et al (1990) Nature 347:737; European patent publication ("EP") 0,344,029). By providing the nuclear genome of the plant with such a male-sterility gene, an artificial nuclear male-sterility locus is created containing the artifical male-sterility genotype that results in a male-sterile plant.

In addition, it has been recently shown that male-fertility can be restored to such a nuclear male-sterile plant with a chimaeric fertility-restorer gene comprising another DNA sequence (or fertility-restorer DNA) that codes, for example, for a protein that inhibits the activity of the cytotoxic product or otherwise prevents the cytotoxic product from being active at least in the selected tissue of the plant's male reproductive organs (EP 0,412,911). For example, the barnase gene of Bacillus amyloliquefaciens codes for an RNase (Barnase) which can be inhibited by a protein (Barstar) that is encoded by the barstar gene of B. amyloliquefaciens. Hence, the barnase gene can be used for the construction of a chimaeric male-sterility gene while the barstar gene can be used for the construction of a chimaeric fertility-restorer gene. Experiments in different plant species (e.g., oilseed rape) have shown that such a chimaeric barstar gene can fully restore the male-fertility of male-sterile lines in which the male-sterility was due to the presence of such a chimaeric barnase gene (EP 0,412,911: Mariani et al (1991) Proceedings of the CCIRC Rapeseed Congress, July 9-11, 1991 Saskatoon, Saskatchewan, Canada; Mariani et al (1992) Nature 357:384). By coupling a marker gene, such as a dominant herbicide resistance gene (for example, the bar gene coding for phosphinothricin acetyl transferase (PAT) that converts herbicidal phosphinothricin to a non-toxic compound [De Block et al (1987) EMBO J. 6:2513]), to the chimaeric male-sterility and/or fertility restorer gene, breeding systems can be implemented to select for uniform populations of male-sterile plants (EP 0,344,029; EP 0,412,911).

The production of hybrid seed of any particular cultivar of a plant species requires the: 1) maintenance of small quantities of pure seed of each inbred parent and 2) the preparation of larger quantities of seed of each inbred parent. Such larger quantities of seed would normally be obtained by several (usually two) seed-multiplication rounds, starting from a small quantity of pure seed ("basic seed") and leading, in each multiplication round, to a larger quantity of seed of the inbred parent and finally to a stock of seed of the inbred parent ("parent seed" or "foundation seed") which is of sufficient quantity to be planted to produce the desired quantities of hybrid seed. Of course, in each seed-multiplication round, larger planting areas (fields) are required.

In order to maintain and enlarge a small stock of seeds of male-sterile plants, it has been necessary to cross the parent male-sterile plants with normal pollen-producing parent plants. The offspring of such a cross will, in all cases, be a mixture of male-sterile and male-fertile plants, and the latter have to be removed from the former. With male-sterile plants containing an artificial male-sterility locus as described above, such removal can be facilitated by genetically linking the chimaeric male-sterility gene to a suitable marker gene, such as the bar gene, which allows the easy identification and removal of the male-fertile plants. EP 0,198,288 and US Patent 4,717,219, by comparison, describe methods for linking such marker genes (which can be visible markers or dominant conditional markers) to endogenous nuclear loci containing male-sterility genotypes.

However, even when suitable marker genes are linked to male-sterility genotypes, the maintenance of parent male-sterile plants still requires the removal from the field of a substantial number of plants. For instance, in systems using a herbicide resistance gene (e.g., the bar gene) linked to a chimaeric male-sterility gene, only half of the parent stock will result in male-sterile plants, thus requiring the removal of the male-fertile plants by herbicide spraying prior to flowering. In any given field, the removal of male-fertile plants effectively reduces the potential yield of hybrid seed or the potential yield of male-sterile plants during each round of seed multiplication for producing of parent seed. This is economically unattractive for many important crop species such as corn and oilseed rape. In order to minimize the number of male-fertile plants which have to be removed, male-fertile maintainer plants have been sought which, when crossed with a male-sterile parent plant, produce a minimum, preferably no, male-fertile offspring, thereby minimizing or avoiding altogether the need to remove such male-fertile offspring. To solve an analogous problem, US Patents 3,710,511 and 3,861,079 have described procedures for producing and maintaining a homogenous population of male-sterile plants by using specific chromosomal abnormalities that are differentially transmitted to the egg and the sperm in the plants.

Summary of the Invention

In accordance with this invention, a cell of a transgenic plant ("the maintainer plant") is provided, in which the nuclear genome contains stably integrated therein: 1) at a first locus or male-sterility locus, a male-sterility genotype in

homozygous condition; and 2) at a second locus or maintainer locus, a maintainer gene in heterozygous condition; the male-sterility locus and the maintainer locus preferably being unlinked; the maintainer gene being a foreign DNA sequence, preferably a foreign chimaeric DNA sequence, containing:

a) a fertility-restorer gene that comprises at least:

i) a fertility-restorer DNA encoding a restorer RNA and/or protein or polypeptide which, when produced or overproduced in some or all of the cells, preferably stamen cells, of the plant, prevents phenotypic expression of the nuclear male-sterility genotype that would render the plant male-sterile in the absence of expression of the fertility-restorer DNA in the some or all stamen cells and

ii) a restorer promoter capable of directing expression of the fertility-restorer DNA at least in the some or all of the cells, preferably stamen cells, of the plant, so that the phenotypic expression of the nuclear male-sterility genotype is prevented, the fertility-restorer DNA being in the same transcriptional unit as, and under the control of, the restorer promoter and

b) a pollen-lethality gene that is selectively expressed in microspores and/or pollen of the plant to produce nonfunctional pollen and that comprises at least:

iii) a pollen-lethality DNA coding for a pollen-lethality RNA and/or protein or polypeptide that, when produced or overproduced in the microspores and/or pollen, significantly

disrupts their metabolism, functioning and/or development and

iv) a pollen-specific promoter capable of directing expression of the pollen-lethality DNA selectively in the microspores and/or pollen of the plant, the pollen-lethality DNA being in the same transcriptional unit as, and under the control of, the pollen promoter.

The cell of the maintainer plant of this invention preferably also comprises, especially in the maintainer locus, at least one first marker gene which comprises at least:

v) a first marker DNA encoding a first marker RNA and/or protein or polypeptide which, when present at least in a first specific tissue or specific cells of the plant, renders the plant easily separable from other plants which do not contain the first marker RNA, protein or polypeptide encoded by the first marker DNA at least in the first specific tissue or specific cells and

vi) a first marker promoter capable of directing expression of the first marker DNA at least in the first specific tissue or specific cells, the first marker DNA being in the same transcriptional unit as, and under the control of, the first marker promoter.

The male-sterility genotype in the cell of the maintainer plant of this invention can be foreign or endogenous but is preferably a foreign, especially chimaeric, male-sterility gene which comprises:

1) a male-sterility DNA encoding a sterility RNA and/or protein or polypeptide which, when produced or overproduced in a stamen cell of the plant in the absence of the restorer RNA, protein or polypeptide,

significantly disturbs the metabolism, functioning and/or development of the stamen cell and

2) a sterility promoter capable of directing expression of the male-sterility DNA selectively in stamen cells of the plant, the male-sterility DNA being in the same transcriptional unit as, and under the control of, the sterility promoter.

The male-sterility genotype in the maintainer plant cell of this invention preferably comprises, especially in the male-sterility locus, at least one second marker gene which comprises at least:

3) a second marker DNA encoding a second marker RNA and/or protein or polypeptide which, when present at least in the second specific tissue or specific cells of the plant, renders the plant easily separable from other plants which do not contain the second marker RNA, protein or polypeptide encoded by the second marker DNA at least in the second specific tissue or specific cells and

4) a second marker promoter capable of directing expression of the second marker DNA at least in the second specific tissue or specific cells, the second marker DNA being in the same transcriptional unit as, and under the control of, the second marker promoter.

Also in accordance with this invention are provided the maintainer plants, the seeds of such plants, and plant cell cultures, all of which consist essentially of the cells of this invention.

Further in accordance with this invention are provided the maintainer gene and plasmids containing the maintainer gene, as well as bacterial host cells (e.g., E. coli or Agrobacterium) containing such plasmids.

Still further in accordance with this invention is provided a process for producing, preferably

enlarging, a homogeneous population of male-sterile plants and their seed that contain a nuclear male-sterility gene in homozygous condition, the process comprising the step of crossing the male-sterile plants with the maintainer plants of this invention. The seed from the resulting male-sterile plants can be harvested and grown into the male-sterile plants. Hybrid seed can then be produced by crossing the male-sterile plants with male-fertile plants of another inbred parent line used as pollinators.

Yet further in accordance with this invention is provided a process for producing, preferably enlarging, a population of the maintainer plants, comprising the step of selfing the maintainer plants.

Detailed Description of the Invention

A male-sterile plant of this invention is a plant of a given species with a nuclear male-sterility genotype.

A restorer plant of this invention is a plant of the same plant species containing, within its nuclear genome, a fertility-restorer gene that is able to restore the male-fertility in offspring which are obtained from a cross between the male-sterile plant and the restorer plant and which contain both the male-sterility genotype and the fertility-restorer gene.

A restored plant of this invention is a plant of the same species that is male-fertile and that contains, within its nuclear genome, the male-sterility genotype and the fertility-restorer gene.

A parent plant or parent of this invention is a plant that can be used for the production of hybrid seed. The female or seed parent plant is the parent from which the hybrid seed is harvested. For the purposes of this invention, the female parent will

always be a male-sterile plant. The male or pollen parent is the parent that is used to fertilize the female parent. In many cases, the male parent will also be a restorer plant.

A line is the progeny of a given individual plant.

The male-sterility genotype of this invention is the genotype of at least one locus, preferably only one locus, in the nuclear genome of a plant (i.e., the male-sterility locus), the allelic composition of which can result in male-sterility in the plant. A male-sterility genotype can be endogenous to the plant, but it is generally preferred that it be foreign to the plant. Preferred foreign male-sterility genotypes are those in which the allele responsible for male-sterility contains a foreign male-sterility gene that comprises:

1) a male-sterility DNA encoding a sterility RNA and/or protein or polypeptide which, when produced or overproduced in a stamen cell of the plant, significantly disturbs the metabolism, functioning and/or development of the stamen cell and

2) a sterility promoter capable of directing expression of the male-sterility DNA selectively in stamen cells of the plant, the male-sterility DNA being in the same transcriptional unit as, and under the control of, the sterility promoter.

Such a male-sterility gene is always a dominant allele at a foreign male-sterility locus. The recessive allele corresponds to the absence of the male-sterility gene in the nuclear genome of the plant.

Preferred foreign male-sterility DNAs and sterility promoters that can be used in the male-sterility genes in female parent plants and maintainer plants of this invention have been described in EP 0,344,029. A particularly useful male-sterility DNA codes for Barnase (Hartley (1988) J.Mol. Biol. 202:913). Particularly useful sterility promoters are tapetum-specific promoters such as: the promoter of the TA29 gene of Nicotiana tabacum (EP 0,344,029) which can be used in tobacco, oilseed rape, lettuce, chicory, corn and other plant species; the PT72, the PT42 and PE1 promoters from rice, the sequences of which are given in SEQ ID no. 7, SEQ ID no. 8, and SEQ ID no. 9, respectively, of the Sequence Listing and which can be used in rice and other plant species (PCT application PCT/EP 92/00274); and the PCA55 promoter from corn, the sequence of which is given in SEQ ID No. 10, which can be used in corn and other plant species (PCT application PCT/EP 92/00275).

A preferred endogenous male-sterility genotype is one in which a recessive allele ("m") in homozygous condition (m/m) at a male-sterility locus produces male-sterility. At a male-sterility locus, male-fertility would otherwise be encoded by a corresponding dominant allele ("M"). Such a male-sterility genotype is known in many plant species (see Kaul (1988) supra; and 1992 issues of Maize Genetics Cooperation Newsletter, published by the Department of Agronomy and U.S. Department of Agriculture, University Of Missouri, Columbia, Missouri, U.S.A.). The DNA sequences in the nuclear genome of a plant corresponding to m and M alleles can be identified by gene tagging, i.e., by insertional mutagenesis using transposons, or by

means of T-DNA integration (see, e.g., Wienand and Saedler (1987) In "Plant DNA Infectious Agents", Ed. by T.H.Hohn and J.Schell, Springer Verlag, New York, p. 205; Shepherd (1988) In "Plant Molecular Biology: a Practical Approach", IRL Press, p. 187; Teeri et al (1986) EMBO J. 5:1755).

Fertility-restorer DNAs and restorer promoters that can be used in the maintainer genes of this invention with a foreign male-sterility genotype have been described in EP 0,412,911. In this regard, fertility-restorer genes in which the fertility-restorer DNA encodes Barstar (Hartley (1988) J.Mol. Biol. 202:913) and is under control of tapetum-specific promoters, such as those described above as sterility promoters, are of particular use. In particular, it is believed that a fertility-restorer DNA coding for a mutant of Barstar, in which the cysteine residue at its position 40 is replaced by serine (Hartley (1989) TIBS 14:450), functions better in restoring the fertility in the restored plants of some species.

When an endogenous male-sterility genotype is homozygous for a recessive allele m, it is preferred that the fertility-restorer gene be the dominant allele M of that male-sterility genotype, preferably under the control of its own promoter. The DNA corresponding to such a dominant allele, including its natural promoter, can be isolated from the nuclear genome of the plant by means of gene tagging as described above.

The pollen-lethality DNAs that are used in the pollen-lethality genes of this invention preferably encode an RNA and/or a protein or polypeptide that, when expressed in microspores or pollen, significantly disrupts their metabolism, functioning

and/or development. In this regard, the pollen-lethality DNAs can encode RNAs, proteins or polypeptides such as are encoded by the male-sterility DNAs described in EP 0,344,029. Of particular interest are male-sterility DNAs that encode ribonucleases (EP 0,344,029) such as RNase T1 from Aspergillus oryzae (Quaas et al (1988) Eur. J. Biochem. 173:617) or Barnase from Bacillus amyloliquefaciens (Hartley (1988) J.Mol.Biol. 202:913).

So that the pollen-lethality DNA is expressed selectively in microspores or pollen of the maintainer plant, it is preferred that the pollen-specific promoter, which controls the pollen-lethality DNA in the pollen-lethality gene, be a promoter capable of directing gene expression selectively in the microspores and/or pollen of the plant. Such a pollen-specific promoter can be an endogeneous promoter or a foreign promoter and can be from the nuclear genome or from the mitochondrial or chloroplast genome of a plant cell, but in any event, the pollen-specific promoter is foreign in the nuclear genome of the plant being transformed. Preferably the pollen-specific promoter causes the pollen-lethality DNA to be expressed only in the microspores and/or pollen, i.e., after meiosis of the microsporocytes in the anthers. The pollen-specific promoter can be selected and isolated in a well known manner from a plant species, preferably the plant species to be rendered male-sterile, so that the pollen-specific promoter directs expression of the pollen-lethality DNA selectively in the microspores and/or pollen so as to kill or disable the microspores and/or pollen in which the pollen-lethality gene is expressed. The pollen-specific

promoter is preferably also selected and isolated so that it is effective to prevent expression of the pollen-lethality DNA in other tissues of the plant. For example, a suitable endogeneous pollen-specific promoter can be identified and isolated in a plant, to be rendered male-sterile, by :

1. searching for an mRNA which is only present in the plant during the development of its microspores and/or pollen;
2. optionally isolating the microspore- and/or pollen-specific mRNA;
3. preparing and isolating a cDNA from the microspore- and/or pollen-specific mRNA;
4. using this cDNA as a probe to identify regions in the plant genome which contain DNA coding for the corresponding microspore- and/or pollen-specific DNA or alternatively using inverse polymerase chain reactions for the geometric amplification of the DNA sequences which flank, upstream and downstream, a chosen core region of the genomic DNA corresponding to the sequence of the microspore- and/or pollen-specific cDNA; and
5. identifying the portion of the plant genome that is upstream (i.e. 5') from the DNA coding for the microspore- and/or pollen-specific mRNA and that contains the promoter of this DNA.

Examples of such pollen-specific promoters are well known (see MacCormick (1991) TIG 7:298). In this regard, Hamilton et al (1989) Sex. Plant Reprod. 2:208 describes a pollen-specific clone ("Zmg13") from maize inbred line W-22, and the use of the promoter sequences of the clone to direct pollen-specific expression in tobacco has been described by Guerrero et al (1990) Mol.Gen.Genet. 224:161). Other pollen-specific promoters that are likewise believed

to be useful are: the promoter of the gene corresponding to the Nicotiana tabacum pollen-specific cDNA NTPc303 described by Weterings et al (1992) Plant Mol. Biol. 18:1101; and the promoter of the gene corresponding to the Brassica napus pollen-specific cDNA B54 described by Shen and Hsu (1992) Mol. Gen. Genet. 234:379.

If the fertility-restorer DNA in the fertility-restorer gene of the maintainer gene is also expressed in microspores and/or pollen at the same time as the pollen-lethality DNA is expressed (due for instance to the activity of the restorer promoter in microspores and/or pollen), it is preferred that the pollen-lethality DNA be different from the male-sterility DNA (the expression of which is intended to be prevented by expression of the fertility-restorer DNA of the maintainer gene). For example, if the male-sterility DNA encodes Barnase in the male-sterile plants to be maintained, the fertility-restorer DNA in the maintainer gene should encode Barstar. Thus, if the restorer promoter in the maintainer gene also directs expression of the fertility-restorer DNA in microspores and/or pollen and at the same time as the pollen-lethality DNA is expressed, the pollen-lethality DNA preferably should not encode Barnase but rather, for example, another RNAse such as RNAse T1.

First and second marker DNAs and first and second marker promoters that can be used in the first and second marker genes of this invention are also well known (EP 0,344,029; EP 0,412,911). In this regard, it is preferred that the first and second marker DNAs be different, although the first and second marker promoters may be the same.

The fertility-restorer gene, the male-sterility gene, the pollen-lethality gene, and the first and second marker genes in accordance with this invention are generally foreign DNA sequences, preferably foreign chimaeric DNA sequences. Such foreign DNA sequences are preferably provided with suitable 3' transcription regulation sequences and polyadenylation signals, downstream (i.e. 3') from their respective fertility-restorer DNA, male-sterility DNA, pollen-lethality DNA, and first and second marker DNAs. In this regard, either foreign or endogenous, transcription termination and polyadenylation signals suitable for obtaining expression of such DNA sequences can be used. For example, the foreign 3' untranslated ends of genes, such as gene 7 (Velten and Schell (1985) Nucl. Acids Res. 13:6998), the octopine synthase gene (De Greve et al (1982) J.Mol. Appl. Genet. 1:499; Gielen et al (1983) EMBO J. 3:835; Ingelbrecht et al (1989) The Plant Cell 1:671), the nopaline synthase gene of the T-DNA region of Agrobacterium tumefaciens Ti-plasmid (De Picker et al (1982) J.Mol. Appl. Genet. 1:561), the chalcone synthase gene (Sommer and Saedler (1986) Mol. Gen. Genet. 202: 429-434), and the CaMV 19S/35S transcription unit (Mogen et al (1990) The Plant Cell 2:1261-1272), can be used.

By "foreign" with regard to a gene or genotype of this invention is meant that the gene or genotype contains a foreign DNA sequence such as a male-sterility DNA, a fertility-restorer DNA, a pollen-lethality DNA, or a marker DNA and/or a foreign promoter such as a sterility promoter, a restorer promoter, a pollen-specific promoter or a marker promoter. By "foreign" with regard to any DNA sequence, such as a coding sequence or a promoter, in a gene or genotype of this invention is meant that such a DNA is not in the same genomic environment in a plant cell, transformed with such a DNA in

accordance with this invention, as is such a DNA when it is naturally found in the cell of the plant, bacteria, animal, fungus, virus or the like, from which such a DNA originates. This means, for example, that a foreign fertility-restorer DNA, male-sterility DNA, pollen-lethality DNA, or marker DNA can be: 1) a nuclear DNA in a plant of origin; 2) endogenous to the transformed plant cell (i.e., from a plant of origin with the same genotype as the plant being transformed); and 3) within the same transcriptional unit as its own endogenous promoter and 3' end transcription regulation signals (from the plant of origin) in the foreign gene or genotype in the transformed plant cell; but 4) inserted in a different place in the nuclear genome of the transformed plant cell than it was in the plant of origin so that it is not surrounded in the transformed plant cell by the genes which surrounded it naturally in the plant of origin. Likewise, a foreign fertility-restorer DNA, male-sterility DNA, pollen-lethality DNA, or marker DNA can also, for example, be: 1) a nuclear DNA in a plant of origin; and 2) endogenous to the transformed plant cell; but 3) in the same transcriptional unit as a different (i.e., not its own) endogenous promoter and/or 3' end transcription regulation signals in a foreign chimaeric gene or genotype of this invention in a transformed plant cell. A foreign fertility-restorer DNA, male-sterility DNA, pollen-lethality DNA, or marker DNA can also, for example, be: 1) a nuclear DNA in a plant of origin; and 2) endogenous to the transformed plant cell; but 3) in the same transcriptional unit as a heterologous promoter and/or 3' end transcription regulation signals in a foreign chimaeric gene or genotype of this invention

in a transformed plant cell. A foreign fertility-restorer DNA, a male-sterility DNA, pollen-lethality DNA, or marker DNA can also, for example, be heterologous to the transformed plant cell and in the same transcriptional unit as an endogenous promoter and/or 3' transcription regulation signals (e.g., from the nuclear genome of a plant with the same genotype as the plant being transformed) in a foreign chimaeric DNA sequence of this invention in a transformed plant cell. Preferably, each fertility-restorer DNA, male-sterility DNA, pollen-lethality DNA, and marker DNA of this invention is heterologous to the plant cell being transformed.

By "heterologous" with regard to a DNA, such as a fertility-restorer DNA, a male-sterility DNA, a pollen-lethality DNA, a marker DNA, a fertility-restorer promoter, a sterility promoter, a pollen-specific promoter or a marker promoter or any other DNA sequence in a gene or a genotype of this invention is meant that such a DNA is not naturally found in the nuclear genome of cells of a plant with the same genotype as the plant being transformed. Examples of heterologous DNAs include chloroplast and mitochondrial DNAs obtained from a plant with the same genotype as the plant being transformed, but preferred examples are chloroplast, mitochondrial, and nuclear DNAs from plants having a different genotype than the plant being transformed, DNAs from animal and bacterial genomes, and chromosomal and plasmidial DNAs from fungal, bacterial and viral genomes.

By "chimaeric" with regard to a foreign DNA sequence of this invention is meant that at least one of its coding sequences : 1) is not naturally found under the control of the promoter present in the

foreign DNA sequence; and/or 2) is not naturally found in the same genetic locus as at least one of its associated marker DNAs. Examples of foreign chimaeric DNA sequences of this invention comprise: a pollen-lethality DNA of bacterial origin under the control of a pollen-specific promoter of plant origin; and a pollen-lethality DNA of plant origin under the control of a pollen-specific promoter of plant origin and in the same genetic locus as a marker DNA of bacterial origin.

By "endogenous" with respect to a gene or genotype of this invention is meant that it is not foreign.

The foreign genes and genotypes of this invention, such as the male-sterility gene and genotype, the fertility-restorer gene and the pollen-lethality gene, can be described like any other genotype: capital letters denote the presence of the foreign genes and genotypes (the dominant allele) while small letters denote their absence (the recessive allele). Hence, in this description of the invention, "S" and "s" will denote the respective presence and absence of the male-sterility gene, "R" and "r" will denote the respective presence and absence of the fertility-restorer gene, and "P" and "p" will denote the respective presence and absence of the maintainer gene.

For an endogeneous male-sterility genotype of this invention, "m" will denote the recessive allele, and "M" will denote the dominant allele. Thus, the recessive allele m in homozygous condition (m/m) at a male-sterility locus would result in male-sterility, and the dominant allele M, when present at a male-sterility locus either in homozygous or heterozygous condition, results in male-fertility.

The cell of a plant, particularly a plant capable of being infected with Agrobacterium such as most dicotyledonous plants (e.g. Brassica napus), can be transformed using a vector that is a disarmed Ti-plasmid containing the male-sterility gene and/or the fertility-restorer gene and/or the pollen-lethality gene and/or the maintainer gene and/or the marker gene(s) of this invention and carried by Agrobacterium. This transformation can be carried out using the procedures described, for example, in EP 0,116,718 and EP 0,270,822. Preferred Ti-plasmid vectors contain a foreign DNA sequence of this invention between the border sequences, or at least located to the left of the right border sequence, of the T-DNA of the Ti-plasmid. Of course, other types of vectors can be used to transform the plant cell, using procedures such as direct gene transfer (as described, for example, in EP 0, 233,247), pollen mediated transformation (as described, for example, in EP 0,270,356, PCT publication WO 85/01856, and US patent 4,684,611), plant RNA virus-mediated transformation (as described, for example, in EP 0,067,553 and US patent 4,407,956) and liposome-mediated transformation (as described, for example, in US patent 4,536,475). Cells of monocotyledonous plants, such as the major cereals including corn, rice, wheat, barley and rye, can be transformed as described in PCT application PCT/EP 91/02198. In case the plant to be transformed is corn, other recently developed methods can also be used such as, for example, the methods described for certain lines of corn by Fromm et al (1990) Bio/Technology 8:833, Gordon-Kamm et al (1990) Bio/Technology 2:603 and Gould et al (1991) Plant Physiol. 95:426. In case the plant to be transformed is rice, recently developed

methods can also be used such as, for example, the method described for certain lines of rice by Shimamoto et al (1989) Nature 338:274, Datta et al (1990) Bio/Technology 8:736 and Hayashimoto et al (1990) Plant Physiol. 93:857.

The so-transformed cell can be regenerated into a mature plant, and the resulting transformed plant can be used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the male-sterility gene, the fertility-restorer gene, the pollen-lethality gene, the marker genes and/or the maintainer gene of this invention in other varieties of the same or related plant species. Seeds obtained from such plants contain the gene(s) of this invention as a stable genomic insert.

The maintainer plant of this invention is of the same species as a male-sterile plant line and can be used for the maintenance of the male-sterile line, i.e. to maintain a homogeneous population of male-sterile plants and a stock of pure seed of the female parent. The maintainer plant of this invention is itself a plant in which male-fertility has been restored and the genome of which contains both a male-sterility genotype and, in the maintainer locus, a fertility-restorer gene of this invention.

If a plant line with a homozygous male-sterility genotype ($A^{m/m}$ or $A^{S/S}$) is available, a maintainer plant for the male-sterile line can be directly obtained by transforming a male-sterile plant of the line with the maintainer gene of this invention and then selecting those transgenic plants which are male-fertility restored plants and in which the maintainer gene is stably integrated in the nuclear genome so that the genetic locus of the male-

sterility genotype and of the maintainer gene are unlinked and segregate independently.

If the male-sterility genotype is foreign to the plant line, alternative strategies can be followed. For example, the maintainer plant of the present invention can be obtained by: transforming a plant cell of the plant line (A) with the maintainer gene of this invention (P); and then regenerating, from the so-transformed plant cell, a transgenic plant containing, stably integrated in its genome, the maintainer gene. Such a transgenic plant ($A^{P/P}$) can then be crossed as a female parent with a plant $A^{S/s,R/r}$ of the same line, which contains at separate loci in its genome a male-sterility gene (S) and a corresponding fertility-restorer gene (R), both in heterozygous condition, but which lacks the maintainer gene. Thus, the cross is in fact: $A^{S/s,R/r,P/p}$ (male) x $A^{s/s,r/r,P/p}$ (female), and the offspring with the genotype $A^{S/s,r/r,P/p}$ (or hereinafter "$A^{S/s,P/p}$" for convenience) are selected and selfed. One eighth of the offspring that have the desired genotype ($A^{S/S,P/P}$) for a maintainer plant of this invention can then be selected. Another eighth of the offspring with the genotype ($A^{S/S,P/P}$) can be used as male-sterile plants to be maintained.

Isolation of plants with desired genotypes can be achieved by means of conventional testcrosses (see, e.g., Fehr (1987) supra), preferably supplemented by detection of the presence of specific genes at the DNA level, e.g., by means of amplification of DNA fragments by the polymerase chain reaction, by Southern blot analysis and/or by phenotypic analysis for the presence and expression of first or second marker genes of this invention.

The cross of a male-sterile plant containing a male-sterility genotype in homozygous condition ($A^{S/S}$ or $A^{m/m}$) with a maintainer plant of this invention ($A^{S/S,P/p}$ or $A^{m/m,P/p}$, respectively) results in a population of seeds that all contain the male-sterility genotype in homozygous condition ($A^{S/S}$ or $A^{m/m}$, respectively) because the maintainer gene is not transmitted through the pollen. This property can be used to advantage in maintaining the basic seed and in the multiplication of basic seed for the final production of parent seed.

The maintainer plants of this invention ($A^{S/S,P/p}$ or $A^{m/m,P/p}$) can themselves be maintained by selfing. The offspring of such selfing will consist of 50% male-fertile maintainer plants ($A^{S/S,P/p}$ or $A^{m/m,P/p}$, respectively) and 50% male-sterile plants containing the male-sterility genotype in homozygous condition ($A^{S/S}$ or $A^{m/m}$, respectively). If desired, the male-sterile plants can be removed either manually on the basis of the male-sterile phenotype or, if the maintainer gene comprises a suitable first marker gene, preferably a first marker gene whose expression confers herbicide resistance to the plant, by using the phenotypic expression of the first marker gene (e.g, by applying herbicide to the offspring so that male-sterile plants that lack the herbicide-resistance gene are killed while maintainer plants with the herbicide-resistance gene survive).

Thus, the maintainer plant of this invention can be easily used to maintain a homogeneous population of male-sterile plants. In this regard, basic seed of a female parent of a given plant species can be crossed with an appropriate male parent to produce hybrid seed. Also, the maintainer plant of this invention can be used economically to multiply the

basic seed of a female parent of a given plant species, so as to obtain sufficient quantities of female parent seed that can be crossed with an appropriate male parent to produce desired quantities of hybrid seed.

A male-sterile line, that is maintained and multiplied by the use of the maintainer plants of this invention, can be used for the production of hybrid seed. In principle, the male-sterile line ($A^{S/S}$) can be crossed directly with another male parent line ($B^{s/s}$) to produce hybrid seed ($AB^{S/s}$). However, as all hybrid plants are male-sterile, no reproduction and no seed set will occur. This is not a problem if the seed is not the harvested product (e.g., with lettuce), but where seed is the harvested product (e.g., with corn and oilseed rape), male-fertility in the hybrid plants should be at least partially restored. This can be accomplished by crossing the male-sterile line with a male-fertile parent line (e.g., $B^{R/R}$) that is also a restorer line, i.e. that also contains a fertility-restorer gene (R). The hybrids produced ($AB^{S/s,R/r}$) will be fully male-fertile. Alternatively the male-sterile-line ($A^{S/S}$) can first be crossed with the male-fertile line ($A^{s/s}$) just prior to hybrid seed productions. This has the advantage of giving a further multiplication of the female parent line. The offspring ($A^{S/s}$) can then be crossed with a suitable male-fertile parent line ($B^{s/s,r/r}$) to produce hybrid seed that is 50% male-fertile. If hybrid seed with 100% male fertility is desired, the offspring can be crossed with a suitable restorer male parent line ($B^{s/s,R/R}$).

In the case of a male-sterile line in which male-sterility is due to an endogeneous male-sterility genotype ($A^{m/m}$) at a male-sterility locus,

hybrid seed can easily be produced by crossing the male-sterile line ($A^{M/m}$) with a line that is homozygous with respect to the endogenous dominant (male-fertility) allele at that male-sterility locus ($B^{M/M}$). All hybrid offspring of this cross will have the genotype $AB^{M/m}$ and will be fertile.

The maintainer plants of this invention can also be used as pollinator (i.e., male-fertile) plants in a cross with wild-type plants ($A^{s/s,p/p}$) of the same inbred line. The progeny of this cross will all be male-sterile and heterozygous for the male-sterility genotype ($A^{S/s,p/p}$). The progeny can therefore be used directly for hybrid seed production by crossing with a pollinator plant line B ($B^{s/s,p/p}$). This scheme only requires a male-sterilization of the wild-type plants, for example by manually removing the anthers (e.g., in corn) or by using a male gametocide.

Of course, by using the maintainer plants of this invention to maintain a homogeneous population of plants that are homozygous with respect to a male-sterility allele (whether dominant or recessive) that is encoded in the nuclear genome, the maintainer plants acquire many of the characteristics of plants of a cytoplasmic male-sterile line. However, such plants do not have one of the major disadvantages of cytoplasmic male-sterile plants, namely the cytoplasmic uniformity of the various male-sterile lines which, in corn, has led to serious problems (see Craig (1977) In "Corn and Corn Improvement", G.F. Sprague, ed., American Society of Agronomy, Inc., Publisher, p. 671).

Thus, the maintainer gene of this invention, when introduced into a particular line of a plant species, can always be introduced into any other line by backcrossing, but since the maintainer gene can

only be transmitted through an egg, it will always be associated with the cytoplasm of the line in which it was initially introduced. However, since a maintainer plant line is only used for maintenance of a male-sterile line and not as a female parent for hybrid seed production, the hybrid seed will always contain the cytoplasm of the female parent, as desired.

The following Examples illustrate this invention. Unless otherwise indicated, all experimental procedures for manipulating recombinant DNA were carried out by the standardized procedures described in Sambrook et al (1989) "Molecular Cloning: A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press, N.Y. USA. All polymerase chain reactions ("PCR") were performed under conventional conditions, using the Vent™ polymerase (Cat. No. 254L - Biolabs New England, Beverley, MA 01915, U.S.A.) isolated from Thermococcus litoralis (Neuner et al (1990) Arch. Microbiol. 153:205-207). Oligonucleotides were designed by the methods described by Kramer and Fritz (1968) Methods in Enzymology 154:350 and synthesized by the phosphoramidite method (Beaucage and Caruthers (1981) Tetrahedron Letters 22:1859) on an applied Biosystems 380A DNA synthesizer (Applied Biosystems B.V., Maarssen, Netherlands).

The following bacterial strains and plasmids, used in the Examples, are available from the Deutsche Sammlung für Mikroorganismen und Zellkulturen ("DSM"), Mascheroder Weg 1B, Braunschweig, Germany:

Bacterial strain	plasmid	DSM No	Date of Deposit
E. coli WK6	pMa5-8	DSM 4567	May 3, 1988
E. coli WK6	pMc5-8	DSM 4566	May 3, 1988

In the Examples, reference will be made to the following Figure and Sequence Listing:

Figure

Figure 1: Ten-step procedure to obtain corn (e.g. H99) maintainer plants of the invention

Sequence Listing

SEQ ID no. 1: genomic DNA comprising the promoter of the Zm13 gene from Zea mays
SEQ ID no. 2: sequence of plasmid "pVE144"
SEQ ID no. 3: sequence of plasmid "pVE108"
SEQ ID no. 4: sequence of oligonucleotide "MDB80"
SEQ ID no. 5: sequence of oligonucleotide "MDB81"
SEQ ID no. 6: sequence of oligonucleotide "MDB82"
SEQ ID No. 7: genomic DNA comprising the anther specific promoter "PT72" from rice
SEQ ID No. 8: genomic DNA comprising the anther specific promoter "PT42" from rice
SEQ ID No. 9: genomic DNA comprising the anther specific promoter "PE1" from rice
SEQ ID No. 10: genomic DNA comprising the anther specific promoter "PCA55" from corn
SEQ ID No. 11: Oligonucleotide Zm13OLI2
SEQ ID No. 12: Oligonucleotide Zm13OLI1
SEQ ID No. 13: Oligonucleotide Zm13OLI5
SEQ ID No. 14: Oligonucleotide BXOL2
SEQ ID No. 15: Oligonucleotide TA29SBXOL2
SEQ ID No. 16: Oligonucleotide PTA29OL5
SEQ ID No. 17: EcoRI-HindIII fragment of pTS218 carrying the maintainer gene.

Examples

Example 1 : Isolation of the pollen-specific promoter of the Zm13 gene from maize.

A pollen-specific cDNA from Zea mays inbred line W-22, designated as "Zmc13", has been isolated and characterized by Hanson et al (1989) The Plant Cell 1:173. The corresponding genomic clone, designated as "Zmg13", containing substantial portions of the 5' flanking region has been isolated and characterized by Hamilton et al (1989) Sex. Plant Reprod. 2:208 (see also Hamilton et al (1992) Plant Mol. Biol. 18:211). The complete sequence of Zmg13 is shown in SEQ ID no. 1, and its promoter region will hereinafter be referred to as the "Zm13 promoter".

A corresponding promoter region from corn inbred line H99 was isolated as follows. Genomic DNA of inbred line H99 was prepared as described by Dellaporta et al (1983) Plant Mol. Biol. Reports 1:19-21. Using the genome as a substrate, a 1471 bp fragment was amplified by PCR using the oligonucleotides MDB80 and MDB82, the sequences of which are shown in SEQ ID no. 4 and SEQ ID no. 6, respectively. MDB80 corresponds to nucleotides 8 to 28 of Zmg13, while MDB82 is complementary to nucleotides 1458 to 1478 of Zmg13. Then, the purified amplified 1471 bp fragment was used as a substrate for the amplification by PCR of a 1422 bp fragment, using the oligonucleotides MDB80 and MDB81. MDB81 is complementary to nucleotides 1409 to 1429 of Zmg13, and its sequence is shown in SEQ ID no. 5. By using MDB81, a NcoI site is created in the amplified 1422 bp fragment at the ATG translation initiation codon.

The 1422 bp fragment is then ligated in an SmaI site of pGEM2 (Promega Corporation, Madison,

Wisconsin 53711, U.S.A.), yielding plasmid pMDB13, and the fragment is sequenced (Maxam and Gilbert (1980) Meth. Enzymol. 65:499). The pollen-specific promoter of the Zm13 gene of corn inbred line H99 is obtained from pMDB13 as a EcoRV-NcoI fragment.

The Zm13 promoter is also cloned as follows. Genomic DNA of Zea mays line H99 is prepared as described above. Using the genomic DNA as a substrate, the following two fragments are amplified by means of PCR: 1) a 875 bp fragment is amplified using the oligonucleotides MDB80 (SEQ ID No. 4) and ZM13OLI2 (which is complementary to nucleotides 859 to 882 of Zmg13 and which sequence is given in SEQ ID No. 11); and 2) a 661 bp fragment is amplified using the oligonucleotides Zm13OLI1 (which corresponds to nucleotides 767 to 791 of Zmg13 and which sequence is given in SEQ ID No. 12) and Zm13OLI5 (which is partially complementary to nucleotides 1397 to 1423 of Zmg13 and which sequence is given in SEQ ID No. 13). The 875 bp fragment, corresponding to the upstream region of the Zm13 promoter, is cloned into the SmaI site of pGEM2, yielding plasmid pTS204. The 661 bp fragment, corresponding to the downstream region of the Zm13 promoter, is digested with NcoI and cloned into plasmid pJB66 (Botterman and Zabeau (1987) DNA 6:583) digested with EcoRV and NcoI, yielding plasmid pTS203. Both fragments partly overlap and share a BstXI site in the region of overlap. Ligation of the 567 bp EcoRV-BstXI fragment of pTS204 and the 638 bp BstXI-NcoI fragment of pTS203 results in a 1205 bp fragment corresponding to the Zm13 promoter. This 1205 bp fragment, as cloned from line H99, is sequenced, and its sequence is found to be identical to the corresponding fragment of Zmg13 from line W-22 as given in SEQ ID No.1

except at position 276 (G in W-22 is T in H99), 410 (G in W-22 is A in H99), and 1205-1206 (GC in W-22 is GGC in H99, thus corresponding to a 1 nucleotide insertion), numberings being as in SEQ ID No. 1.

Example 2 : Construction of plant transformation vectors comprising a maintainer gene that contain DNA encoding Barstar under the control of the TA29 promoter and DNA encoding Barnase under the control of the Zm13 promoter.

The 1205 bp EcoRV-NcoI fragment of pMDB13 is ligated to the large EcoRI-SmaI fragment of plasmid pVE144 and to the 739 bp EcoRI-NcoI fragment of pVE108, yielding plasmid pGSJVR1. Plasmid pVE144, the sequence of which is shown in SEQ ID no. 2, is a plasmid derived from plasmid pUC18 (Yanisch-Perron et al (1985) Gene 33:103) and containing DNA encoding neomycin phosphotranferase (neo) under the control of the 35S3 promoter (EP 0,359,617) from Cauliflower Mosaic Virus isolate CabbB-JI (Hull and Howell (1978) Virology 86:482) and DNA encoding the Barstar (Hartley (1988) J.Mol.Biol. 202:913) under the control of the tapetum-specific promoter of the TA29 gene of Nicotiana tabacum (EP 0,344,029; Seurinck et al (1990) Nucleic Acids Res. 18:3403). Plasmid pVE108, the sequence of which is shown in SEQ ID no. 3, is a plasmid derived from pUC18 and containing DNA encoding phosphinothricin acetyl transferase (bar) (EP 0,242,236) under the control of the 35S3 promoter and DNA encoding Barnase (Hartley (1980) supra) under the control of the TA29 promoter. The resulting plasmid, pGSJVR1 (which is subsequently renamed "pTS210"), is a pUC18-derived plasmid that contains a maintainer gene of this invention comprising: DNA encoding Barnase as the pollen-lethality DNA, the Zm13 promoter as the pollen-specific promoter, DNA

encoding Barstar as the fertility-restorer DNA, the TA29 promoter as the restorer promoter, neo as the first marker DNA and the 35S3 promoter as the first marker promoter.

pTS210 is also obtained as follows. The 0.9 kb BstXI-SacI fragment of pTS204 is ligated to the large BstXI-SacI fragment of pTS203, yielding plasmid pTS206. The 1.47 BglII-NcoI kb fragment ofpTS206 is then ligated to the large NcoI-BglII fragment of pVE108, yielding plasmid pTS207. Finally, the 1.9 kb EcoRV-Eco-RI fragment of pTS207 is ligated to the large Eco-RI-SmaI fragment of pVE144, yielding plasmid pTS210.

A plasmid pTS218, which differs from pTS210 by carrying the bar gene as a selectable marker gene, is also obtained as follows:

- a 255 bp DNA fragment, designated as bxx and carrying the translation initiation site of the PTA29-barstar gene, is obtained by PCR using pVE144 as a template and oligonucleotides BXOL2 (SEQ ID No. 14) and TA29SBXOL2 (SEQ ID No. 15) as primers.
- a 492 bp DNA fragment is prepared by PCR using pVE108 and bxx as a template and oligonucleotides PTA29OL5 (SEQ ID No. 16) and BXOL2 as primers. This 492 bp fragment is digested with AsnI and BspEI, and a 274 bp fragment is purified on gel and ligated to the 6.28 kb fragment of pVE144 which was digested with BspEI and partially digested with AsnI. The resulting plasmid is designated as pVEK144 and carries the PTA29-barstar-3'nos chimeric gene

with an optimized translational initiation context.

- pVEK144 is digested with MunI and HindIII, and the 3.7 kbp fragment is isolated and ligated to the 1.7 kbp MunI-HindIII fragment of pVE108, yielding plasmid pVEB144 which carries the PTA29-barstar-3'nos and the P35S-bar-3'nos chimeric genes.
- theEcoRI-HindIII fragment of pVEB144, containing the two chimeric genes, is ligated to the large EcoRI-HindIII fragment of pUCNew2, yielding plasmid pVEC144. pUCNew2 is derived from pUC19 as described in WO 92/13956.
- finally, the large EcoRI-SmaI fragment of pVEC144 is ligated to the 1.9 bp EcoRV-EcoRI fragment of pTS207, yielding plasmid pTS218.

Plasmid pTS218 carries three chimeric genes, i.e., PTA29-barstar-3'nos (with optimized translational initiation context), P35S-bar-3'nos, and PZM13-barnase-3'nos. The EcoRI-HindIII fragment of pTS218 carrying these three chimeric genes is presented in the sequence listing as SEQ ID No. 17.

All steps of vector construction involving fragments of the barnase DNA, such as pVE108, pVE144, and ptS210, are carried out in E. coli strain MC1061 containing the cointegrate plasmid R702::pMc5BS which is obtained as follows. Plasmid pMc5BS, containing the barstar gene (encoding an inhibitor of barnase) under the control of the tac promoter (De Boer et al (1983) Proc. Natl. Acad. Sci. USA 80:21), is constructed by: cloning the EcoRI-HindIII fragment of plasmid pMT416 (Hartley (1988) supra) into the EcoRI and HindIII sites of plasmid pMc5-8 (DSM 4566); and then deleting the sequence starting with the initiation codon of the phoA signal sequence and ending with the last nucleotide before the

translation initiation codon of the barstar-coding region by means of a looping-out mutagenesis procedure as generally described by Sollazo et al (1985) Gene 37:199.

Plasmid R702 is from Proteus mirabilis and can replicate in E.coli (Villarroel et al (1983) Mol. Gen. Genet. 189:390). Plasmid R702::pMc5BS is obtained by cointegration through illegitimate recombination between pMc5BS and R702, mediated by transposable elements present on R702 (Leemans (1982) "Technieken voor het gebruik van Ti-plasmieden van Agrobacterium tumefaciens als vectoren voor de genetic engineering van planten", Ph.D. Thesis Vrije Universiteit Brussel, Brussels, Belgium) and checked for induced expression of Barstar.

The use of E.coli (R702::pMc5BS) allows the construction, maintenance, amplification, and purification of plasmids containing the barnase DNA, such as pGSJVR1, without any lethal effect on the host due to accidental expression of the barnase DNA. However, because the Zm13 promoter is not expressed in E. coli, all steps of vector construction involving this promoter are also carried out in E. coli strain MC1061.

Example 3 : Transformation of corn with the maintainer gene of Example 2.

Zygotic immature embryos of about 0.5 to 1 mm are isolated from developing seeds of corn inbred line H99. The freshly isolated embryos are enzymatically treated for 1-2 minutes with an enzyme solution II (0.3% macerozyme (Kinki Yakult, Nishinomiya, Japan) in CPW salts (Powell & Chapman (1985) "Plant Cell Culture, A Practical Approach", R.A. Dixon ed., Chapter 3) with 10% mannitol and 5 mM 2-[N-Morpholino] ethane sulfonic acid (MES), pH 5.6).

After 1-2 minutes incubation in this enzyme solution, the embryos are carefully washed with N6aph solution (macro- and micro-elements of N6 medium (Chu et al (1975) Sci. Sin. Peking 18:659) supplemented with 6mM asparagine, 12 mM proline, 1 mg/l thiamine-HCl, 0.5 mg/l nicotinic acid, 100 mg/l casein hydrolysate, 100 mg/l inositol, 30 g/l sucrose and 54 g/l mannitol). After washing, the embryos are incubated in the maize electroporation buffer, EPM-KCl (80 mM KCl, 5 mM $CaCl_2$, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and 0.425 M mannitol, pH 7.2). Approximately 100 embryos in 200 µl EPM-KCl are loaded in each electroporation cuvette. About 20 µg of a plasmid DNA, pPGSJVR1 (of Example 2) linearized with EcoRI, is added per cuvette.

After 1 hour DNA incubation with the explants, the cuvettes are transferred to an ice bath. After 10 minutes incubation on ice, the electroporation is carried out: one pulse with a field strength of 375 V/cm is discharged from a 900 µF capacitor. The electroporation apparatus is as described by Dekeyser et al (1990) The Plant Cell 2:591. Immediately after electroporation, fresh liquid N6aph substrate is added to the explants in the cuvette, after which the explants are incubated for a further 10 minute period on ice.

Afterwards, the embryos are transferred to Mah1 VII substrate (macro- and micro-elements and vitamins of N6 medium supplemented with 100 mg/l casein hydrolysate, 6 mM proline, 0.5 g/l MES, 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 2% sucrose solidified with 0.75 g/l $MgCl_2$ and 1.6 g/l Phytagel (Sigma Chemical Company, St Louis, Mo. U.S.A.), pH 5.8) and supplemented with 0.2M mannitol. After 3 days, the embryos are transferred to the same substrate supplemented with 200 mg/l kanamycin. After

approximately 14 days, the embryos are transferred to Mahl VII substrate without mannitol, supplemented with kanamycin. The embryos are further subcultured on this selective substrate for approximately 2 months with subculturing intervals of about 3 weeks. The induced embryogenic tissue is carefully isolated and transferred to MS medium (Murashige and Skoog (1962) Physiol. Plant 15:473) supplemented with 5 mg/l 6-benzylaminopurine for line H99. The embryogenic tissue is maintained on this medium for approximately 14 days and subsequently transferred to MS medium without hormones and 6% sucrose for line H99. Developing shoots are transferred to 1/2 MS medium with 1.5% sucrose for further development to normal plantlets. These plantlets are transferred to soil and cultivated in the greenhouse.

In an analogous way, corn embryos are transformed with a fragment of pTS218 DNA which contains the maintainer gene and the chimeric P35S-bar-3'nos and which is obtained by digestion of the plasmid with EcoRI, XhoI and PstI and by purifying the longest fragment. Transformation and plant regeneration is as described in Example 5.

Example 4 : Analysis of the transgenic corn plants of Example 3.

Plants form Example 3 transformed with pGSJVR1 are analysed for the presence of the maintainer gene by means of PCR. DNA is prepared according to the protocol described by Dellaporta et al (1983) Plant Mol. Biol. Reporter 1:19, adapted for application to tissue amounts of about 10 to 20 mg. For each plant, such an amount of tissue is macerated in extraction buffer in a microfuge tube. Representative fragments of the maintainer gene are amplified using appropriate oligonucleotide probes.

Activity of the expression product of the first marker gene (i.e., neomycin phosphotransferase II (NPTII)) is assayed in plants as follows. Crude extracts are prepared by grinding plant tissue in extraction buffer (McDonnell et al (1987) Plant Molecular Biol. Reporter 5:380). The extracts are then subjected to non-denaturing polyacrylamide gel electrophoresis according to the procedure described by Reiss et al (1984) Gene 30:211. NPTII activity is then assayed by in situ phosphorylation of kanamycin using [gamma-32P]ATP as a substrate (McDonnell et al (1987) supra).

The plants that are found to be positive on both the PCR and NPTII assay are further analyzed by means

of Southern hybridization. Genomic DNA is prepared from plant tissue according to the protocol described by Dellaporta et al (1983) supra, supplemented by a treatment with RNase to remove remaining RNA. A non-transformed H99 plant is used as a control. Samples of the DNA are digested with appropriate restriction enzymes and subjected to horizontal agarose electrophoresis. Southern transfer to Hybond N+ (Amersham International PLC, Amersham, United Kingdom) membranes by means of the "alkali blotting of DNA" protocol and the subsequent hybridization are performed as recommended by the manufacturer (Amersham Hybond-N+ leaflet). Suitable radioactive probes are prepared with the multi-prime DNA labelling kit (Amersham) according to the protocol supplied by the manufacturer which is derived from published procedures (Feinberg and Vogelstein (1983) Anal. Biochem. 132:6). The banding patterns show that at least the maintainer gene is integrated into the plant genomic DNA.

The PCR assays show that the maintainer gene is present. The NPTII assays show that the first marker DNA is expressed. The mature transformed plants can then be analyzed phenotypically to see whether the barstar DNA is expressed in tapetum cells and the barnase gene is expressed in pollen cells. Expression of barstar is determined by northern blotting of anther mRNA and by making testcrosses to determine the restoration in the progeny. Expression of the pollen-lethality gene is determined by cytological examination of the anther. In this regard, viable and nonviable mature pollen is determined by analyzing the staining of isolated pollen upon incubation for 30 minutes at 24°C in the following reaction mixture: 100 mM phosphate buffer pH 7.8, 100 mM

Sodiumsuccinate and 1 mM NitroBlue Tetrazolium, followed by visual inspection of formazan precipitation in viable pollen. Alternative techniques for the differentiation between viable and nonviable mature pollen are those described for example by Alexander (1969) Stain Technology 44:117, and by Heslop-Harrison and Heslop-Harrison (1970) Stain Technology 45:115. The viability of microspores is determined by embedding flower buds in plastic at different developmental stages and subjecting the buds to histochemical staining with the succinate dehydrogenase assay, both as described by De Block and Debrouwer (1992) The Plant Journal 2:261.

Ultimately, the progeny of the plant transformed with the pollen-lethality gene is determined. None of the offspring obtained from a cross using this plant as a male parent have this gene, while 50% of the offspring obtained from a cross using this plant as a female parent possess the gene.

Plants from Example 3, transformed with pTS218 DNA, are analyzed in the same way, except that the expression product of the first marker gene, i.e., phosphinothricine acetyltransferase, is assayed by means of a PAT assay as described in Example 5.

Example 5 : Production of male-sterile corn plants.

Zygotic embryos of corn inbred line H99 were isolated, enzymatically treated, washed, and loaded in electroporation buffer as described in Example 3. Approximately 100 embryos in 200 µl EPM-KCl were loaded in each electroporation cuvette. About 20 µg of a plasmid DNA, pVE108 linearized with HindIII, was added per cuvette. pVE108 is a 5620 bp plasmid which contains: a chimaeric gene comprising the bar DNA (EP 242236), encoding phosphinothricin acetyl transferase (PAT) and conferring resistance to an herbicidal glutamine synthetase inhibitor such as phosphinothricin (PPT), under the control of the 35S3 promoter; and another chimaeric gene comprising the DNA coding for barnase (Hartley (1988) supra) under the control of the tapetum-specific promoter of the

TA29 gene (EP 344029) of N. tabacum. The complete sequence of plasmid pVE108 is given in SEQ ID no. 4. All vector constructions involving DNA fragments comprising the barnase gene were carried out in E. coli strain MC1061 containing the plasmid R702::pMc5BS of Example 3. After a 1 hour DNA incubation with the explants, the cuvettes were transferred to an ice bath. After 10 minutes incubation on ice, the electroporation was carried out as described in Example 3. Immediately after electroporation, fresh liquid N6aph substrate was added to the explants in the cuvette, after which the explants were incubated for a further 10 minute period on ice.

Afterwards, the embryos from one electroporation experiment were transferred to Mah1 VII substrate supplemented with 0.2 M mannitol and 2 mg/l PPT. After approximately 14 days, the embryos were transferred to Mh1 VII substrate (Mah1 VII substrate of Example 3 but without proline and casein hydrolysate) supplemented with 2 mg/l PPT but without mannitol. After approximately 4 weeks, the embryos were subcultured for another month on Mh1 VII substrate supplemented with 10 mg/l PPT. The induced embryogenic tissue was carefully isolated and transferred to MS medium supplemented with 5 mg/l 6-benzylaminopurine. The embryogenic tissue was maintained on this medium for approximately 14 days and subsequently transferred to MS medium without hormones and sucrose. Developing shoots were transferred to 1/2 MS medium with 1.5% sucrose for further development to normal plantlets. These plantlets survived an in vitro spraying with doses of BASTA^R (Hoechst AG, Frankfurt am Main, Germany) corresponding to 2 l/ha. These plantlets were then

transferred to soil and cultivated in the greenhouse, and two of the transformed plantlets, designated RZM35-1 and RZM35-18, were further characterized.

The embryos from a second electroporation experiment were transferred to Mh1 VII substrate supplemented with 2 mg/l PPT and 0.2 M mannitol. After about 14 days, the embryos were transferred to Mh1 VII substrate supplemented with 2 mg/l PPT but without mannitol. After approximately another three weeks, the embryos were transferred to Mh1 VII substrate supplemented with 10 mg/l PPT but without mannitol. After another three weeks, the induced embryogenic tissue was carefully isolated and transferred to MS medium supplemented with 2 mg/l PPT and 5 mg/l 6-benzylaminopurine. The embryogenic tissue was maintained on this medium for approximately 14 days and subsequently transferred to MS medium without hormones, sucrose or PPT. Developing shoots were transferred to 1/2 MS medium with 1.5% sucrose for further development to normal plantlets. The resulting plantlets were transferred to soil and cultivated in the greenhouse, and three of the transformed plantlets, designated RZM34-1, RZM34-12, and RZM34-14, were further characterized.

RZM34-1, RZM34-12, RZM34-14, RZM35-1, and RZM35-18 were grown in the greenhouse. Activity of the expression product of the bar gene in leaves of the plants was assayed as follows in a "PAT assay". 100 mg of leaf tissue from each plant, together with 50 mg of acid-treated sea sand (Merck, Darmstadt, Germany) and 5 mg polyvinylpolypyrrolidone (PVPP), were ground in an Eppendorf tube with a glass rod in 50 µl of extraction buffer (25 mM Tris-HCL pH 7.5, 1 mM Na_2-EDTA (ethylenediaminetetraacetic acid disodium salt), 0.15 mg/ml phenylmethylsulfonylfluoride

(PMSF), 0.3 mg/ml dithiothreitol (DTT), and 0.3 mg/ml bovine serum albumin). The extract was centrifuged in a microfuge for 5 minutes at 16000 rpm. The supernatant was recovered and diluted ten times with TE 25/1 (25 mM Tris-HCL pH 7.5, 1 mM Na_2-EDTA. To twelve µl of the diluted extract was then added: 1µl of 1 mM PPT in TE 25/1, 1 µl of 2 mM AcetylCoenzyme A in TE 25/1, and 2 µl of [14C]AcetylCoenzym A (60 mCi/mmol, 0.02 mCi/ml, [NEN Research Products, Dupont, Wilmington, Delaware, USA). The reaction mixture was incubated for 30 minutes at 37°C and spotted on a aluminium sheet silicagel 60 t.l.c. plate with concentrating zone (Merck). Ascending chromatography was carried out in a 3 to 2 mixture of 1-propanol and NH_4OH (25% NH_3). 14C was visualized by overnight autoradiography (XAR-5 Kodak film). The tolerance to the herbicide BASTA^R was tested by brushing a small area near the top of one leaf per plant with a 1% solution of the herbicide and observing the damage symptoms at and near the brushed sites. While RZM34-1, RZM35-1 and RZM35-18 showed no damage symptoms at all, RZM34-12 and RZM34-14 displayed slight browning and drying-out of the brushed site. RZM34-1, RZM34-12, RZM34-14, RZM35-1 and RZM35-18 were also shown to be male-sterile but otherwise phenotypically completely normal; female fertility, for instance, was normal. The spikelets of the male flowers were of about normal length but were very thin and appeared to be empty, and they never opened. A detailed analysis showed that the anthers were reduced to almost microscopic structures. This phenotype indicates not only that at least one copy of the barnase gene was expressed but also that it was selectively expressed in some or all of the tissues of the anthers.

Southern analysis showed RZM35-1 and RZM35-18 to have an identical integration pattern, with only one copy of plasmid pVE108 being present in the genome of each plant. A small part of the plasmid DNA sequence adjacent to the HindIII site (used for linearization prior to electroporation) seemed to be absent in the integrated copy. Southern analysis of RZM34-1, RZM34-12 and RZM34-14 showed that each of these plants probably has two or three copies of part or all of pVE108 integrated into its genome. The copies are most likely not inserted in a concatemer configuration.

Transformants RZM35-1 and RZM34-1 were pollinated with pollen from an untransformed H99 plant, and progeny plantlets were recovered. From the 35 plantlets recovered from RZM35-1, 16 (46%) scored positive in a PAT assay, while 19 (54%) were PAT negative. This proportion in the F1 progeny does not differ significantly from the 1:1 ratio expected under normal Mendelian segregation of one active copy of the chimaeric bar gene ($X2 = 0.26$).

From the 34 plantlets recovered from RZM34-1, 19 (56%) scored positive in a PAT assay, while 15 (44%) were PAT negative. This proportion in the F1 progeny does not differ significantly from the 1:1 ratio expected under normal Mendelian segregation, assuming that the transformed female parent had one active copy, or alternatively multiple active, but closely linked copies, of the chimaeric bar gene ($X2 = 0.47$).

Example 6 : Production of restorer corn plants.

Zygotic embryos of corn inbred line H99 were isolated, enzymatically treated, washed and loaded in electroporation buffer as described in Example 5. Approximately 100 embryos in 200 µl EPM-KCl were loaded in each electroporation cuvette. About 20 µg

of a plasmid DNA, pVE144 linearized with HindIII, was added per cuvette. pVE144 is a 6555 bp plasmid which was described in Example 2.

The embryos were electroporated, and the transformed cells were selected, grown into callus, and regenerated as described in Example 3. Transgenic plants were analyzed for the presence of the fertility-restorer gene and the marker gene by means of Southern hybridization and PCR. The expression of the fertility-restorer gene is assayed by means of Northern blotting, and the expression of the marker gene is determined by NPTII assay as described in Example 3.

Example 7 : Production of maintainer corn plants and a male-sterile corn line and maintenance of the male-sterile corn line

Maintainer plants of this invention of corn line H99 are obtained as outlined in Figure 1. A plant of corn inbred line H99 with the male-sterility genotype $H99^{S/s,r/r,p/p}$, transformed with the male-sterility gene of Example 5, is crossed with plants with the genotype $H99^{s/s,R/r,p/p}$, transformed with the fertility-restorer gene of Example 6. The progeny that have the genotype $H99^{S/s,R/r,p/p}$ are identified by PCR analysis for the presence of the S and R genes. These plants are selfed, yielding progeny with nine different genotypes. Two of these genotypes ($H99^{S/S,r/r}$ and $H99^{S/s,r/r}$) will develop into male-sterile plants, while all the other genotypes will develop into male-fertile plants. When these male-fertile plants are selfed, progeny analysis allows the identification of their genotype. Thus: a) the progeny of selfings of $H99^{S/S,R/R}$, $H99^{S/s,R/R}$, $H99^{s/s,R/R}$, $H99^{s/s,R/r}$ and $H99^{s/s,r/r}$ would all develop into male-fertile plants; b) selfings of $H99^{S/s,R/r}$ plants would

produce progeny, of which 13 out of 16 would be male-fertile, and since the male-sterility gene is linked to the herbicide resistance gene, bar, 4 out of the 13 male-fertile plants would be sensitive to the herbicide BASTA^R ; and c) selfings of H99$^{S/S,R/r}$ plants would produce progeny, of which 12 out 16 would be fertile (4 out of 16 would have the genotype H99$^{S/S,R/R}$ and 8 out of 16 would have the genotype H99$^{S/S,R/r}$), all of which would be resistant to the herbicide, and the male-sterile progeny of which (4 out of 16) would all be homozygous for the male-sterility gene (H99$^{S/S,r/r}$).

The homozygous male-sterile progeny (H99$^{S/S,r/r}$) of selfing (c) are then crossed with their male-fertile siblings, and only when the cross is with plants with the genotype H99$^{S/S,R/r}$ are the resulting plants 50% male-sterile (all with the genotype H99$^{S/S,r/r}$) and 50% male-fertile (all with the genotype H99$^{S/S,R/r}$. Indeed, the alternative cross between H99$^{S/S,r/r}$ and H99$^{S/S,R/R}$ would result in 100% male-fertile progeny plants.

Maintainer plants are selected by crossing the plant with the genotype H99$^{S/s,R/r,P/p}$ with a plant that is heterozygous for the maintainer gene of Example 2, i.e., (H99$^{s/s,r/r,P/p}$), using the latter plant as the female parent. The offspring with the genotype H99$^{S/s,r/r,P/p}$ are selected by means of testcrosses supplemented with PCR analysis of the progeny (which can be easily identified by PCR and Southern blotting for the presence of the S and P genes and the absence of the R gene). The selected fertile offspring are then selfed. One out of eight offspring have the desired genotype for a maintainer plant of this invention (H99$^{S/S,P/P}$) and can be further selected by means of testcrosses and PCR analysis of the

progeny. Indeed, only plants with this genotype will produce 50% male-sterile offspring (all $H99^{S/S,P/P}$) and 50% male-fertile offspring (all $H99^{S/S,P/P}$), thus growing at once both the desired homozygous male-sterile line and the maintainer line of this invention. Testcrosses also include the pollination of wild type H99 plants with pollen of the progeny plants obtained from the selfing of $H99^{S/s,P/P}$ plants.

Homozygous male-sterile plants with the genotype $H99^{S/S,r/r,P/P}$ are then pollinated by maintainer plants ($H99^{S/S,r/r,P/P}$) of this invention. All progeny have the genotype $H99^{S/S,r/r,P/P}$, so that the male-sterile line is maintained, as desired.

Example 8 : Introduction of the male-sterility gene and the maintainer gene in inbred corn lines through classical breeding.

The male-sterility gene of Example 5 and the maintainer gene of Example 2 are transferred from corn inbred line H99 to another corn inbred line (A) by repeated backcrossings as follows. The maintainer plant $H99^{S/S,P/P}$ is crossed as a female parent with an untransformed plant of line A ($A^{s/s,P/P}$). The offspring with the genotype $A\text{-}H99^{S/s,P/P}$ are selected by screening, using PCR, for the presence of both the maintainer gene (P) and the male-sterility gene (S). These plants are then crossed again as female parents with $A^{s/s,P/P}$ plants, and the offspring that are heterozygous for both the P and S genes are again selected by PCR. This process of backcrossing is repeated until finally plants with the genotype $A^{S/s,P/P}$ are obtained. These plants are then selfed, and the progeny are analyzed in the same way as

described in Example 7. In this way, male-sterile plants with the genotype $A^{S/S,P/P}$ and maintainer plants of this invention with the genotype $A^{S/S,P/P}$ are obtained.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(C) CITY: Ghent
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(I) TELEX: 11.361 Pgsgen

(ii) TITLE OF INVENTION: Maintenance of male-sterile plants

(iii) NUMBER OF SEQUENCES: 17

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/899,072
(B) FILING DATE: 12-JUN-1992

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/970,849
(B) FILING DATE: 03-NOV-1992

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2661 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Zea mays
(B) STRAIN: inbred line W-22

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Hamilton et al.,
(C) JOURNAL: Sex Plant Reprod.
(D) VOLUME: 2
(F) PAGES: 208-
(G) DATE: 1989

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6555 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: plasmid pVE144 (replicable in E.coli)

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..396
(D) OTHER INFORMATION: /label= pUC18
/note= "pUC18 derived sequence"

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(B) LOCATION: complement (397..751)
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(A) NAME/KEY: -
(B) LOCATION: complement (1025..1607)
(D) OTHER INFORMATION: /label= TA29
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(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1608..2440
(D) OTHER INFORMATION: /label= 35S3
/note= "35S3 promoter sequence derived from cauliflower mosaic virus isolate CabbB-JI"

(ix) FEATURE:
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(B) LOCATION: 2441..3256
(D) OTHER INFORMATION: /label= neo
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(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 3257..4315
(D) OTHER INFORMATION: /label= 3'ocs
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(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 4316..6555
(D) OTHER INFORMATION: /label= pUC18
/note= "pUC18 derived sequence"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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AATTCTGTTG TGCACGTTGT AAAAAACCTG AGCATGTGTA GCTCAGATCC TTACCGCCGG 3540

TTTCGGTTCA TTCTAATGAA TATATCACCC GTTACTATCG TATTTTTATG AATAATATTC 3600

TCCGTTCAAT TTACTGATTG TACCCTACTA CTTATATGTA CAATATTAAA ATGAAAACAA 3660

TATATTGTGC TGAATAGGTT TATAGCGACA TCTATGATAG AGCGCCACAA TAACAAACAA 3720

TTGCGTTTTA TTATTACAAA TCCAATTTTA AAAAAAGCGG CAGAACCGGT CAAACCTAAA 3780

AGACTGATTA CATAAATCTT ATTCAAATTT CAAAAGGCCC CAGGGGCTAG TATCTACGAC 3840

ACACCGAGCG GCGAACTAAT AACGTTCACT GAAGGGAACT CCGGTTCCCC GCCGGCGCGC 3900

ATGGGTGAGA TTCCTTGAAG TTGAGTATTG GCCGTCCGCT CTACCGAAAG TTACGGGCAC 3960

CATTCAACCC GGTCCAGCAC GGCGGCCGGG TAACCGACTT GCTGCCCCGA GAATTATGCA 4020

GCATTTTTTT GGTGTATGTG GGCCCCAAAT GAAGTGCAGG TCAAACCTTG ACAGTGACGA 4080

CAAATCGTTG GGCGGGTCCA GGGCGAATTT TGCGACAACA TGTCGAGGCT CAGCAGGGGC 4140

TCGATCCCCT CGCGAGTTGG TTCAGCTGCT GCCTGAGGCT GGACGACCTC GCGGAGTTCT 4200

ACCGGCAGTG CAAATCCGTC GGCATCCAGG AAACCAGCAG CGGCTATCCG CGCATCCATG 4260

CCCCCGAACT GCAGGAGTGG GGAGGCACGA TGGCCGCTTT GGTCGACCTG CAGCCAAGCT 4320

TGGCGTAATC ATGGTCATAG CTGTTTCCTG TGTGAAATTG TTATCCGCTC ACAATTCCAC 4380

ACAACATACG AGCCGGAAGC ATAAAGTGTA AAGCCTGGGG TGCCTAATGA GTGAGCTAAC 4440

TCACATTAAT TGCGTTGCGC TCACTGCCCG CTTTCCAGTC GGGAAACCTG TCGTGCCAGC 4500

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ACTCAAAGGC GGTAATACGG TTATCCACAG AATCAGGGGA TAACGCAGGA AAGAACATGT 4680

GAGCAAAAGG CCAGCAAAAG GCCAGGAACC GTAAAAAGGC CGCGTTGCTG GCGTTTTTCC 4740

ATAGGCTCCG CCCCCCTGAC GAGCATCACA AAAATCGACG CTCAAGTCAG AGGTGGCGAA 4800

ACCCGACAGG ACTATAAAGA TACCAGGCGT TTCCCCCTGG AAGCTCCCTC GTGCGCTCTC 4860

CTGTTCCGAC CCTGCCGCTT ACCGGATACC TGTCCGCCTT TCTCCCTTCG GGAAGCGTGG 4920

CGCTTTCTCA ATGCTCACGC TGTAGGTATC TCAGTTCGGT GTAGGTCGTT CGCTCCAAGC 4980

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GATTATCAAA AAGGATCTTC ACCTAGATCC TTTTAAATTA AAAATGAAGT TTTAAATCAA 5460

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GAAGTGGTCC TGCAACTTTA TCCGCCTCCA TCCAGTCTAT TAATTGTTGC CGGGAAGCTA 5760

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TGGTGTCACG CTCGTCGTTT GGTATGGCTT CATTCAGCTC CGGTTCCCAA CGATCAAGGC 5880

GAGTTACATG ATCCCCCATG TTGTGCAAAA AAGCGGTTAG CTCCTTCGGT CCTCCGATCG 5940

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CTCTTACTGT CATGCCATCC GTAAGATGCT TTTCTGTGAC TGGTGAGTAC TCAACCAAGT 6060

CATTCTGAGA ATAGTGTATG CGGCGACCGA GTTGCTCTTG CCCGGCGTCA ATACGGGATA 6120

ATACCGCGCC ACATAGCAGA ACTTTAAAAG TGCTCATCAT TGGAAAACGT TCTTCGGGGC 6180

GAAAACTCTC AAGGATCTTA CCGCTGTTGA GATCCAGTTC GATGTAACCC ACTCGTGCAC 6240

CCAACTGATC TTCAGCATCT TTTACTTTCA CCAGCGTTTC TGGGTGAGCA AAAACAGGAA 6300

GGCAAAATGC CGCAAAAAAG GGAATAAGGG CGACACGGAA ATGTTGAATA CTCATACTCT 6360

TCCTTTTTCA ATATTATTGA AGCATTTATC AGGGTTATTG TCTCATGAGC GGATACATAT 6420

TTGAATGTAT TTAGAAAAAT AAACAAATAG GGGTTCCGCG CACATTTCCC CGAAAAGTGC 6480

CACCTGACGT CTAAGAAACC ATTATTATCA TGACATTAAC CTATAAAAAT AGGCGTATCA 6540

CGAGGCCCTT TCGTC 6555

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5620 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: plasmid pVE108 (replicable in E.coli)

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..395
(D) OTHER INFORMATION: /label= pUC18
/note= "pUC18 derived sequence"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (396..802)
(D) OTHER INFORMATION: /label= 3'nos
/note= "3' regulatory sequence containing the polyadenylation site derived from the nopaline synthase gene from Agrobacterium T-DNA"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (803..1138)
(D) OTHER INFORMATION: /label= barnase
/note= "coding region of the barnase gene of Bacillus amyloliquefaciens"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1139..1683)
(D) OTHER INFORMATION: /label= TA29
/note= "sequence derived from tapetum specific promoter of Nicotiana tabacum"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1684..2516
(D) OTHER INFORMATION: /label= 35S3

/note= "'35S3" promoter sequence derived from cauliflower mosaic virus isolate CabbB-JI"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 2517..3068
(D) OTHER INFORMATION: /label= bar
/note= "coding sequence of phosphinotricin acetyltransferase gene"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 3069..3356
(D) OTHER INFORMATION: /label= 3'nos
/note= "3' regulatory sequence containing the polyadenylation site derived from Agrobacterium T-DNA nopaline synthase gene"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 3357..5620
(D) OTHER INFORMATION: /label= pUC18
/note= "pUC18 derived sequence"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA 60

CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG TCAGCGGGTG 120

TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC 180

ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC 240

ATTCGCCATT CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT 300

TACGCCAGCT GGCGAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGGTA ACGCCAGGGT 360

TTTCCCAGTC ACGACGTTGT AAAACGACGG CCAGTGAATT CGAGCTCGGT ACCCGGGGAT 420

CTTCCCGATC TAGTAACATA GATGACACCG CGCGCGATAA TTTATCCTAG TTTGCGCGCT 480

ATATTTTGTT TTCTATCGCG TATTAAATGT ATAATTGCGG GACTCTAATC ATAAAAACCC 540

ATCTCATAAA TAACGTCATG CATTACATGT TAATTATTAC ATGCTTAACG TAATTCAACA 600

GAAATTATAT GATAATCATC GCAAGACCGG CAACAGGATT CAATCTTAAG AAACTTTATT 660

GCCAAATGTT TGAACGATCT GCTTCGGATC CTCTAGAGNN NNCCGGAAAG TGAAATTGAC 720

CGATCAGAGT TTGAAGAAAA ATTTATTACA CACTTTATGT AAAGCTGAAA AAAACGGCCT 780

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TATAGTTAAT ATCCGCTTCA CGCCATGTTC GTCCGCTTTT GCCCGGGAGT TTGCCTTCCC 960

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TATGATATGT CTGAAGATAA TCCGCAACCC CGTCAAACGT GTTGATAACC GGTACCATGG 1140

TAGCTAATTT CTTTAAGTAA AAACTTTGAT TTGAGTGATG ATGTTGTACT GTTACACTTG 1200

CACCACAAGG GCATATATAG AGCACAAGAC ATACACAACA ACTTGCAAAA CTAACTTTTG 1260

TTGGAGCATT TCGAGGAAAA TGGGGAGTAG CAGGCTAATC TGAGGGTAAC ATTAAGGTTT 1320

CATGTATTAA TTTGTTGCAA ACATGGACTT AGTGTGAGGA AAAAGTACCA AAATTTTGTC 1380

TCACCCTGAT TTCAGTTATG GAAATTACAT TATGAAGCTG TGCTAGAGAA GATGTTTATT 1440

CTAGTCCAGC CACCCACCTT ATGCAAGTCT GCTTTTAGCT TGATTCAAAA ACTGATTTAA 1500

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CCTTCTTGTT TGGCACTATA TTCAATCTGT TAATGCAAAT TATCCAGTTA TACTTAGCTA 1680

GATCCTACGC AGCAGGTCTC ATCAAGACGA TCTACCCGAG TAACAATCTC CAGGAGATCA 1740

AATACCTTCC CAAGAAGGTT AAAGATGCAG TCAAAAGATT CAGGACTAAT TGCATCAAGA 1800

ACACAGAGAA AGACATATTT CTCAAGATCA GAAGTACTAT TCCAGTATGG ACGATTCAAG 1860

GCTTGCTTCA TAAACCAAGG CAAGTAATAG AGATTGGAGT CTCTAAAAAG GTAGTTCCTA 1920

CTGAATCTAA GGCCATGCAT GGAGTCTAAG ATTCAAATCG AGGATCTAAC AGAACTCGCC 1980

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TGGGCTCCAC GCTCTACACC CACCTGCTGA AGTCCCTGGA GGCACAGGGC TTCAAGAGCG 2880

TGGTCGCTGT CATCGGGCTG CCCAACGACC CGAGCGTGCG CATGCACGAG GCGCTCGGAT 2940

ATGCCCCCCG CGGCATGCTG CGGGCGGCCG GCTTCAAGCA CGGGAACTGG CATGACGTGG 3000

GTTTCTGGCA GCTGGACTTC AGCCTGCCGG TACCGCCCCG TCCGGTCCTG CCCGTCACCG 3060

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TACGTTAAGC ATGTAATAAT TAACATGTAA TGCATGACGT TATTTATGAG ATGGGTTTTT 3240

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TGGGGTGCCT AATGAGTGAG CTAACTCACA TTAATTGCGT TGCGCTCACT GCCCGCTTTC 3540

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CCTGGAAGCT CCCTCGTGCG CTCTCCTGTT CCGACCCTGC CGCTTACCGG ATACCTGTCC 3960

GCCTTTCTCC CTTCGGGAAG CGTGGCGCTT TCTCAATGCT CACGCTGTAG GTATCTCAGT 4020

TCGGTGTAGG TCGTTCGCTC CAAGCTGGGC TGTGTGCACG AACCCCCCGT TCAGCCCGAC 4080

CGCTGCGCCT TATCCGGTAA CTATCGTCTT GAGTCCAACC CGGTAAGACA CGACTTATCG 4140

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GCTCTGCTGA AGCCAGTTAC CTTCGGAAAA AGAGTTGGTA GCTCTTGATC CGGCAAACAA 4320

ACCACCGCTG GTAGCGGTGG TTTTTTTGTT TGCAAGCAGC AGATTACGCG CAGAAAAAAA 4380

GGATCTCAAG AAGATCCTTT GATCTTTTCT ACGGGGTCTG ACGCTCAGTG GAACGAAAAC 4440

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GTTGCCTGAC TCCCCGTCGT GTAGATAACT ACGATACGGG AGGGCTTACC ATCTGGCCCC 4680

AGTGCTGCAA TGATACCGCG AGACCCACGC TCACCGGCTC CAGATTTATC AGCAATAAAC 4740

CAGCCAGCCG GAAGGGCCGA GCGCAGAAGT GGTCCTGCAA CTTTATCCGC CTCCATCCAG 4800

TCTATTAATT GTTGCCGGGA AGCTAGAGTA AGTAGTTCGC CAGTTAATAG TTTGCGCAAC 4860

GTTGTTGCCA TTGCTACAGG CATCGTGGTG TCACGCTCGT CGTTTGGTAT GGCTTCATTC 4920

AGCTCCGGTT CCCAACGATC AAGGCGAGTT ACATGATCCC CCATGTTGTG CAAAAAAGCG 4980

GTTAGCTCCT TCGGTCCTCC GATCGTTGTC AGAAGTAAGT TGGCCGCAGT GTTATCACTC 5040

ATGGTTATGG CAGCACTGCA TAATTCTCTT ACTGTCATGC CATCCGTAAG ATGCTTTTCT 5100

GTGACTGGTG AGTACTCAAC CAAGTCATTC TGAGAATAGT GTATGCGGCG ACCGAGTTGC 5160

TCTTGCCCGG CGTCAATACG GGATAATACC GCGCCACATA GCAGAACTTT AAAAGTGCTC 5220

ATCATTGGAA AACGTTCTTC GGGGCGAAAA CTCTCAAGGA TCTTACCGCT GTTGAGATCC 5280

AGTTCGATGT AACCCACTCG TGCACCCAAC TGATCTTCAG CATCTTTTAC TTTCACCAGC 5340

GTTTCTGGGT GAGCAAAAAC AGGAAGGCAA AATGCCGCAA AAAAGGGAAT AAGGGCGACA 5400

CGGAAATGTT GAATACTCAT ACTCTTCCTT TTTCAATATT ATTGAAGCAT TTATCAGGGT 5460

TATTGTCTCA TGAGCGGATA CATATTTGAA TGTATTTAGA AAAATAAACA AATAGGGGTT 5520

CCGCGCACAT TTCCCCGAAA AGTGCCACCT GACGTCTAAG AAACCATTAT TATCATGACA 5580

TTAACCTATA AAAATAGGCG TATCACGAGG CCCTTTCGTC 5620

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide MDB80

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..21
(D) OTHER INFORMATION: /label= MDB80
/note= "oligonucleotide designated as MDB80"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCGCTTGTCA GTGAATGTTG C 21

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide MDB81

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..21
(D) OTHER INFORMATION: /label= MDB81
/note= "oligonucleotide designated as MDB81"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CCGAGGCCAT GGTTGCCGCC G 21

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide MDB82

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..21
(D) OTHER INFORMATION: /label= MDB82
/note= "oligonucleotide designated as MDB82"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ACGCATAGGC ATAGGATGAC G 21

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3627 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oryza sativa
(B) STRAIN: Akihikari

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..2845
(D) OTHER INFORMATION: /label= PT72
/note= "sequence comprising anther specific promoter PT72"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 2733..2739
(D) OTHER INFORMATION: /label= TATA
/note= "TATA Box"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 2765
(D) OTHER INFORMATION: /note= "transcription initiation determined by primer extension"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 2846..2848
(D) OTHER INFORMATION: /label= ATG
/note= "ATG start of translation of rice T72 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GACAATACAT CAAGTAAATC AAACATTACA AATCAGAACC TGTCTAAGAA TCCATCTTAA 60

TTCAGAAAAA AACTCAGATT AGATGTTCAT GCTTCCACCA GAAGCAGGAA TGTGCAACCT 120

ACACTTCCTG TAATTTCCAT ACTACAATGT CCCCACTGAC CACTGTGCCT GATGCTCTAT 180

TAGAATACCA CATCCTCCAT GGCTCCATGT AAATGCATAT AAATTTGACT CTTTAAATTA 240

GTAACTACAA TTTAAAATTT ATCGAACATT GTTCAAATTT ATAAACAGTT TCCCCAAATT 300

TAGATGCTCC CAAATGTACA CAGCTACTAG TAAAGCACCA TCCAGTTTCA CCTGAACAGG 360

ACTGACATAA ATGTGTGAAA AGGGGACGTC ATTCCCCCAA ATACAACTGA ACAATCCTCC 420

ATCAGAACAT TCATTTGATT GACATTACTC GGAGAGATAC AGCTCGCAGG CACACGAGAT 480

TCTTCTGCCT TTCCAATTGC CACGAACCCA CATGTCACAC GACCAACCAA AAAGAGAGAA 540

TTTTTCTTTG CACAAACAAA AAGTGAGATT TTTTTTTCGC CACAAAGGTG CGAACTTTCT 600

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GCTCCCAAGT CAAACTGATG GAGACCACCT ACTATCTTCC TCTTGTTTTC TTCTGCTTTT 960

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GGTATCAGAA TCCTAATCTG TTAGCTCAGC ATTTGTTTTT GTGTGTTTAA TTGGCCATGA 1140

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GTTGCAAACA ACAAACAGGT CCTTCCAAAC GGGTTGGTTT CATTCCACAG AACAGGATAG 1260

CAACCAGAGC ACAAACCGTT CAACAATATA TATATATATA TATATATATA TATATATATA 1320

TATATATATA TATATATATG ATTTAAAATT ATATTACTAT TTTTAGGATA CGGAACTCTT 1380

AACACATGAA AATCTAAACA TTTTCAACCA ATCAGAACTA CTAGAAAGAT AATCTAACTA 1440

CTTCAAAATT TAAAATTTGA CAAATAAAAT AACTAGTTTT TTCTAAAGCT ATCTTCACTG 1500

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TATTCTTCTT CTCATGAAAA ATTAACTTCT CGCTATAAAT CCGAACATAT ATTATGCGTA 1620

GCAAGTTGTT TTTTTTAACG GGTGGAGTAA TATTAGAGTA TTTAAATTCC TTCAAATTGC 1680

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TTGCATCCTT TCCAGCTCGT AGCTACTGTT CTGCATGTAT TGGACTTGGA TTAAGATCAA 1800

ATGCAGTTGC TATTGTAACT GCACAATAGC AACTGCACAC AATCATGTCC ATTCGTTTTC 1860

AGATCCAACG GCTCTAGATG ACTGCTACAG TACATGCATA ATAGTACATC TCTGCTACAG 1920

TGTTTTTGCT GCAGTACCAC TTCATATCCT GGCCTTCCGT TCTAGATCAT GTGATGTACA 1980

TGTTTTTTTG AAACAACCCG CACAAGACAT TGATAGAGTA GGAAATGTGA TGTACATGTT 2040

AACGGCTTAA GTTACAGTTA CAATAACAAC TGCACAGGAT CTTGATCCAT TGGACTTGTA 2100

TAATATCTCA TCTCGTCGTT CCATTATCGT GGTAACAGTT GGCAACTTGG CATCCAGTGC 2160

TGGAAACTAT GCCGTGTGTA CATCAGGATC GTCCTTTTTG TTCAGTTCCA AGATAGAACA 2220

AGTCCAAAAG ATGGCCGTAG TTTTTTTAGT CACAGTGGAA GCTGACATAG CCGTGGAATA 2280

AGTTCTGCAC AAAAGTTGCC ATTCGAGATC AACTACTGGT AGTAGTAGTC ATCTTCTACC 2340

ACTGCGAATA TTCGAAGGGA CACAAAAAGA TCAACGAGTA AATTAGTTCA CCGGAAGACG 2400

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CGCGGGCGCC GACCGCGCGT CCGCCGCATG CGCGCGGCGT AGGTGAGCAA CGCGGGCCTC 2700

GCCGCGCGCG CTCCCCTCCT TCGATCCCCT CCTATAAATC GAGCTCGCGT CGCGTATCGC 2760

CACCACCACC ACGACACACA CGCACGCACC GTGCAGGCAT CGACGACGAG CGAGAGCCCC 2820

TCGGCGGCAG AAGACACTCA CGGCGATGGC GGTGACGAGG ACGGCGCTGC TGGTGGTGTT 2880

GGTAGCGGGG GCGATGACGA TGACGATGCG CGGGGCGGAG GCGCAGCAGC CGAGCTGCGC 2940

GGCGCAGCTC ACGCAGCTGG CGCCGTGCGC GCGAGTCGGC GTGGCGCCGG CGCCGGGGCA 3000

GCCGCTGCCG GCGCCCCCGG CGGAGTGCTG CTCGGCGCTG GGCGCCGTGT CGCACGACTG 3060

CGCCTGCGGC ACGCTCGACA TCATCAACAG CCTCCCCGCC AAGTGCGGCC TCCCGCGCGT 3120

CACCTGCCGT AAGAAAACGA ATAAAATCGA TTTGCTATCT ATCGATGATT GTGTTTTTGT 3180

AGACTAAACT AAACCCCTAT TAATAATCAA CTAACCGATG AACTGATCGT TGCAGAGTGA 3240

TGGAGATGGT GTGCCAAGGT AATTGCGTTT GCTCGTGCGA GGATGAGAAG AGAAGATTGA 3300

ATAAGATGTT TGATGGCAAC AAGTCATCAG GCGATCCGAT CCCTGCAGCT ATGAATGGGA 3360

GTATACGTAG TAGTGGTCTC GTTAGCATCT GTGTGTCGCA TATGCACGCC GTGCGTGCCG 3420

TGTCTGTCCT GCTTGCTCTG CTGATCGTTC AATGAACGAC AAATTAATCT AACTCTGGAG 3480

TGACAAGTCG TTCGAGATAT ACTAATACTA CCATGTGCAG GGTCTTTCAA CCAAGGTTCA 3540

TGTTTTCCAC GAAAGCCGAT TGAAACGAAA CCGCGAAATT TTGATGCGAG ATGAAAGCAG 3600

ATTCCGAGTG AAATTTTAAA TGGTTTT 3627

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2370 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oryza sativa
(B) STRAIN: Akihikari

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..1808
(D) OTHER INFORMATION: /label= PT42
/note= "sequence comprising anther specific promoter PT42"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1748..1755
(D) OTHER INFORMATION: /label= TATA

/note= "TATA Box"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1780
(D) OTHER INFORMATION: /note= "transcription initiation site determined by primer extension"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1809
(D) OTHER INFORMATION: /label= ATG
/note= "ATG start of translation of rice T42 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGCCATCACT GTCGGGTGCT GCGCCATGGA CATCACCGTC TCCTTCCTGC GCCGCCGTCG 60

CCGGTGAGCT CCAAGGCCGA AGCCTTCTTC CCCTCACGCC ACTACCTCTC TCTTCCCCAA 120

TTCCGGCCAA CGCCGTCCGT TGCCACAGCG CCACCTCCAC GCCATCCCAG AGCCCCGTGC 180

CGTGCCACCG GGTTCGCCTC CATCTCCTCT TGCCAACGCC GACGCTCGTC GCGGCAGCCA 240

TGCGCTGTCA CCGATGAACA CCGCCGCGCC ACAGCCATGG CAGAGCACGG CCAGGGAGCC 300

ATGGCTGCTC TGCCTCCTCC TCCTTCTCTC ACATCTGGTT GCAGCCGGAC CTAGTCGGCT 360

TATACAAATG GCCCATGGGC AAAATTGTCT TTTATGAAAG TTTCTCTCAC CGTTTCAGTC 420

GGAAATAATA AAATAATGGG AGGATTGTCC GCCAGCAAAT TACCATATTT TTTCGGTGTC 480

CAAGAGCAAA TACACGATCT TCGGGTGTTT CACAGCAAAG ACCACAATTT CTAAGTGTCC 540

TGTAACAAAT TTTGCCAATA AAAATTTAAA ACCAAAGGAG AAGACTGTAC ATGAAGAAAA 600

ACAAAGAGAA TGAAATTACA TAAGCTCAGG GGTTATAAAG TTGATTTATT TTTAGGATGA 660

AGGAAGTGTG TGAAAACAAT GGCCAATTGG GTGTCGGAAA ATATAACGTG CTTGCTAAAA 720

TGTCGTCCCC ATATCCTGTA GCTGATTATA GATAGACCCT GATGGTCAAG ATGCCCTGTA 780

CTGGATCGTG TTTCCATGCT TCATCTCCGC TTCTCTCAAG TACTCCCCGA ACTCACATAT 840

CTGGTGGGCT GGATCCACAG TAAGAAACAG TCAAACAACA CTCACTTCAT AGATAACCAA 900

TTGTTTAATT ATTCTTAGTC CCTTATCTTA TACTCCTAGT AAGTGCTTAA AAACTTGGTA 960

TAAATATCAA ATTTATCGTA CAATTACAAT ATAATTATAA CGTATACCAT GTAATTTTTA 1020

AAACTATTTT TAGATAAAAA AAATATGGTG ATGAGCAGCC GCAGCAGCGG ACGCCGAACC 1080

ACCTGCCGAA CATCACCAAG ATAGCGAGTC CTAAAAATTT TTAGTGTTCG TTTGCTGGGT 1140

TGGTAACTAA TTAAAAAAAA AGAGCGACTC ATTAGCTCAT AAATAATTAC GTATTAGCTA 1200

ATTTTTTTAA AAAATAAATT AATATAACTT ATAAAGCAGC TTTTGTATAA TTTTTTTTTT 1260

AAAAAAGTGT TGTTTAGCAG TTTTGGGAAG TGTGCCGAGG GAAAACGATG AGATGGGTTG 1320

GGGAAGGAGG GGGAAGAAGT GAAGAACACA GCAAATATAG GCAGCATCGT CCCGTACAGA 1380

TCAGGCTGCA ACCACGCCCC GCGGAGATAG TTAACGCGGC CCACGTTGTG CTATAGCCCG 1440

TCACTCTCGC GGGCCTCTCC AACCTCCAGT TTTTTTTCTA GCCCATCAGC TGATACGGGG 1500

CCTTCCCCCC ATGCAGGAGG ATGGCCCGCC ACGCGGTGTT TTGGGCCGTT CTCGCCGCGC 1560

GCGCCCGTGC CGATCCGGGA CTCATCCCAC GTGCCGCCTC GCCACCGCCG CCGCCGCCGC 1620

TGCTGCTCCG GCTGCCGGCT GGACCTTCAC GCTCACGCGC TCTCCCCTGC CCAACCACCA 1680

CGCAAACAAA CACGAAGTTC GCGCCGTCGA CCGGCTCCCC TCCTCCCCCG CGCGCATCGG 1740

ATCCCCCTAC ATAAACCCTC TCGCTCGCCA TCGCCATGGC AGCAACTCCC CTCCTCCACT 1800

AGACCACCAT GCACAGATCG ATGGCCTCTC AGGCGGTGGC GCCCCTCCTC CTCATCCTCA 1860

TGCTCGCGGC GGCGGCGGGG GGCGCGTCGG CGGCGGTGCA GTGCGGGCAG GTGATGCAGC 1920

TGATGGCGCC GTGCATGCCG TACCTCGCCG GCGCCCCCGG GATGACGCCC TACGGCATCT 1980

GCTGCGACAG CCTCGGCGTG CTCAACCGGA TGGCCCCGGC CCCCGCCGAC CGCGTCGCCG 2040

TCTGCAACTG CGTCAAGGAC GCCGCCGCCG GCTTCCCCGC CGTCGACTTC TCCCGCGCCT 2100

CCGCCCTCCC CGCCGCCTGC GGCCTCTCCA TCAGCTTCAC CATCGCCCCC AACATGGACT 2160

GCAACCAGTA AGTTCATTCA TTCTTTCTTA ACTCCAATTC AATTTATCCA TCACCTCGAC 2220

TTAAGCCTGA TTAAACTTAA CTTGTTCTTT GCATGCTTGC ACTATTGCAG GGTTACAGAG 2280

GAACTGAGAA TCTGAGAGCG TGAGGAATCG AGTTCATGTT GCATTTATCA TCAATCATCA 2340

TCGACTAGAT CAATAAATCG AGCAAAGCTT 2370

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2407 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oryza sativa
(B) STRAIN: Akihikari

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..2263
(D) OTHER INFORMATION: /label= PE1
/note= "sequence comprising anther specific promoter PE1"

(ix) FEATURE:
(A) NAME/KEY: -

(B) LOCATION: 2181..2187
(D) OTHER INFORMATION: /label= TATA
/note= "TATA Box"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 2211
(D) OTHER INFORMATION: /note= "transcription initiation site determined by primer extension"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 2264..2266
(D) OTHER INFORMATION: /label= ATG
/note= "ATG start of translation of E1 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TGATAGTGAC ATACTCACAT GCTTTGTCAA TTCAAGTATC AGTTCTTTTC ATATTGATTT 60

CTTAGTTGAT GAAAGTATAC ATATTTCTTG CCATCAATTC TTTTAGTAGG TACATTTGGA 120

CACTAGTGGT CAGGGTTGAA CTCTTAACTG GAGTCTCATC TGATTTGCTT ATCTGAGACT 180

GGGTTTGTGC AAATCCTGTC ATGAGGCAAG GTGGACTGTC AGTCCATGAC ACTTTGCTAC 240

TTCTATTAAG TTCTCGAAAT CTTTTCCAGT GTATGTCCGT TCTCTTTCAA ATGAATTATT 300

TATATGTTCT GACAGCCTCG CGGTGTACAT TTCATTTAAC TTTTGTCTTC ACAGGGCCTC 360

TTGGTATTTT GTTGAGCAGA TTGGAATCAA CCTTCTTGTA GAACTTCTTG ATGTCGTCGC 420

TACCCTTTGC AACTAGATGG TCAACTTCTG TCTTATATCT TTGGTACAAC ACTGGCAAAG 480

TGTGCGCGCA CAAGAATCCT GTGAAGTAAG AAATACAAAC TTGTCATTGT GAAAGTTTAG 540

CTTTATATGA TCTTGACTCT AAATTGTTTC TCCTCAGATC CTTCTGTGTG ATTGTTTTAT 600

TAAAATTTAA TATTTATCTG GAATACCTAC CAATATATAG TAGACTTGTC AAGCTGCAAG 660

AACTTCCAAT CGCCGACAAT ACCAATAGAG ATCCAACCAC CTTAATATCA TAAACAATCT 720

GATTGTTAGT CCAGAACTAT ATTGAGTAGT GAACAACAAT AGCACATTAA CATTATGAGG 780

ATTATTGGCT AACTCTGCAA TTCAATATTC TGATGCGTCT AATCTGGTCA ATTTTAGCGC 840

TCCAGAAAGA ATTGCACAAT CCTTGGACAA TGTTGGCACT GGAACTGTTG CATGTTTTTA 900

CATCTCTTAT TAACGTAGCA AAGGAGTAGA TTATTATGTA CCAGGAGAAA TCTCTTCAGA 960

TCCTTTCCAC ATGCAATGTC GTAAAGAACA GATACAGTGT ACGTTAGTTT GTAATGGACG 1020

GTCAATGCCA TTTCTCTGAA GGCATGTTCA GAGATGATGA TTTCTGGGAT CCTTGGAGGG 1080

GCCCTGAAAT TCGGAAACAG TTAGTTGAGT TTTAGTACCT AATGTCTTGC GTTATACTAC 1140

GTGAAATGCC ATTTCTGTAA GCTGAGTTTT CTACCATCTC CACAGGAAAT AAAGCTAATA 1200

CCTGTCCAAG AGTGGTGCGG CATTTGACCA AATGAAGATC ACAAGCATGG CAAGAATGGC 1260

AATCTGGCAA AGGAGCGGAA TTATATTGTA TTCTACTACA TCGAACAGGA ACCATATCAA 1320

TGTTGCCCCA GCAAGGACCC CCGCAGATAA GTTCCTGTTC TTCCACAGCA GAATATCCGC 1380

AACTGCATAG CTCCCAACAA TGAAATCCAA AACCACATCG GCTCAGAGAG AAGTTATGAT 1440

AAAAGGCACT AATTCTGAAT AATTTCCTAG AAAGCGAATA ATAATAGCAC ACCTTGACCT 1500

CCACCAAGAA GCTTGTGGAT CGACTTGTGC CCATGAAATG GCATTCTGAC ATTCTGGTCA 1560

CTGTCAGAAT CTCTCGGAAA ATGAGGAGGC ATAGCTTCGT GTGTGTATGT GTGTGGGATA 1620

TTACGCTGCT AAAACTTTGT GTTTCTGATC GATCTGGTTA GAGAGCATCG TCTTTATAAG 1680

CACTTAAAAA TGGTAGTATA ATCTCTCAAG GAGCCTATAC TGCCAAGGAA AGGATAGCTT 1740

GGCCTGTGGG GATTGAGCCG TTGAAGGGAA CAAACGAATA CAGTTACCTT ACCAGATGTT 1800

TGCCACGACA TGGGCAACGT CATTGCTAGA CCAAGAAGGC AAGAAGCAAA GTTTAGCTGT 1860

CAAAAAAGAT ATGCTAGAGG CTTTCCAGAA TATGTTCTAT CTCAGCCAGA CCAATGGGGG 1920

CAAAATTTAC TACTATTTGC CATACATTAA CCACGTAAAA GTCCTACACT CAACCTAACT 1980

GTTGAACGGT CCTGTTCTGG CCAACGGTGA GAATGCACCT AATGGACGGG ACAACACTTC 2040

TTTCACCGTG CTACTGCTAC ATCCTGTAGA CGGTGGACGC GTGAGGTGCT TTCGCCATGA 2100

CCGTCCTTGG TTGTTGCAGT CACTTGCGCA CGCTTGCACC GTGACTCACC TGCCACATTG 2160

CCCCCGCCGT CGCCGGCGCC TACAAAAGCC ACACACGCAC GCCGGCCACG ATAACCCATC 2220

CTAGCATCCC GGTGTCCAGC AAGAGATCCA TCAAGCCGTC GCGATGACGA CGAGGCCTTC 2280

TGTTTTTTCC ACCGTTGTCG CGGCGATCGC CATCGCCGCG CTGCTGAGCA GCCTCCTCCT 2340

CCTGCAGGCT ACCCCGGCCG CGGCCAGCGC GAGGGCCTCG AAGAAGGCTT CGTGCGACCT 2400

GATGCAG 2407

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2784 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Zea mays

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..1179
(D) OTHER INFORMATION: /label= PCA55
/note= "region comprising the anther specific promoter and the leader sequence, PCA55"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1072
(D) OTHER INFORMATION: /label= TATA
/note= "TATA Box"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1180..1596
(D) OTHER INFORMATION: /note= "presumed coding sequence of corn CA55 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TGGTATGCAT CAATAGAGCC GGAAGATGGT CTGGAGTAAG GACCTGGCAG TGTGATACGG 60

GAACTTGACA TCTGAATAGA TATTCTCCCT TGTCCCTCTG GTAAAAAAAA CTGTTGTCAC 120

ATTTGCCTTC GCTGTGACTT GGATGTATCA TGTATATCTT TGACCATTGA TATCTTGGTT 180

AATCAGACGG TGCATTACAA TCATGGCCTC ATTCATATAG GGTTTAGGGT TACCACGATT 240

GGTTTGCATA AGTAGTACCC CTCCGTTTCA AATTATGTCG TATTTTGATT TTTTAGATAC 300

ACTTTTTATA TAATTTTTTA TTTTAAATTA GGTGTTTTAT ATAATACGTA TCTAAGTGTA 360

TAATAAAATA TATGTATCTA AAAGCTGTAA TTTAGTATAA ATTAGAATGG TGTATATCTT 420

CAATGTATGA CAAATAATTT GAAATGGAGG AGGGTATGAA AAGCCAAAAC CTCCTAGAAT 480

ATGGAATGGA GGGAATACAT ACAAATTCTT TGCTTCAGTT AAAAGAAACG AGAAAAGGAG 540

GGGAATGGGG AATCGTACTT CAGTTTTTAC GAGTTTTCAT CAAACATGTA TGCACGTCTT 600

CCCTTGGTTG ATGCATCTTT TTGGCAAATC TTCGTTTAAT TGCGGCTTCT TTTTTATACC 660

GTTCGAAGGT TTTCGTCGTC AATGCTGAAA CTCCACTTTC ACCACCTTCG GTTGCATCTG 720

CTTGCTTTCA ATTCACCTCT AATTAGTCCA AGTGTTTCAT TGGACGAAGG TCCAAGTCCT 780

TCAGATCATC TCAATTTTCT TTGATCTGAA ACAACAATTT AAAACTGATT TTGTTACCTT 840

GACCTGTCGA AGACCTTCGA ACGAACGGTA CTGTAAAAAT ACTGTACCTC AGATTTGTGA 900

TTTCAATTCG ATTCGGGTCT CCTGGCTGGA TGAAACCAAT GCGAGAGAAG AAGAAAAAAT 960

GTTGCATTAC GCTCACTCGA TCGGTTACGA GCACGTAGTT GGCGCCTGTC ACCCAACCAA 1020

ACCAGTAGTT GAGGCACGCC CTGTTTGCTC ACGATCACGA ACGTACAGCA CTATAAAACA 1080

CGCAGGGACT GGAAAGCGAG ATTTCACAGC TCAAAGCAGC CAAAACGCAG AAGCTGCACT 1140

GCATATACAG AAGATACATC GAGCTAACTA GCTGCAGCGA TGTCTCGCTC CTGCTGCGTC 1200

GCCGTGTCGG TGCTTCTCGC TGTCGCCGCG ACAGCCAGCG CCACCGCGCC GGCATGGCTG 1260

CACGAGGAGC AGCACCTCGA GGAGGCCATG GCCACGGGCC CGCTGGTCGC AGAGGGTGCG 1320

AGGGTGGCGC CCTCCGCGTC CACCTGGGCT GCCGACAAGG CGTCGCCGGC GAGGCCGAGC 1380

GGCGGCATGG CCACGCAGGG CGACGACCAG AGCTCGTCGG GCGGCAGTGG CAGCAGCGGT 1440

GAGCACGGCA AGGCGGAGGG CGAGAAGCAG GGCAAGAGCT GCCTCACCAA GGAGGAGTGC 1500

CACAAGAAGA AGATGATCTG TGGCAAGGGC TGCACGCTCT CGGCGCACAG CAAGTGCGCC 1560

GCCAAGTGCA CCAAGTCCTG TGTCCCCACC TGCTAGGAGC CGAGGCCGGA GCTTGCCGGC 1620

GGCGAGACCT CGATCGATCG AGTGCTTCAC TTCACTTCTT TGTTATAGTT CTTGTGTGTT 1680

GCCGTTGCGT TGCGTTGCGT AGACGAAGGG AATAAGGAAG GGTAATTGGA TTACCTGTTC 1740

CAGATCTCTG TGTAAGCGTG TTGTCGTGAC AAGTCTTTTG ATCCAGAGCG AGGGATGGAT 1800

AGATCGCGCT CGCAGTTTTA ATTGCAATGC TAGTTCAATA TGTGTGCATC ATGTTGGCAA 1860

CTACATAGTC CAGATTCAAA CCGAGATCGC TGTTTAGCAT GCCAGCACAA TAATAACGGT 1920

ACAATCATAT TATATTTTAT ACAAATGCAC AATTTATCTC TAGAGATGTC AATGGGAAAT 1980

TCCTCATCGG GTTATATCAT CTCAGACTCA TCCCCATCAT ATTTGATTCA TCCTCATACT 2040

CATCCTCATA TCTATCATGA GTGCAAAACT CATTTCATAC CCATCTCTAT TTTGGTTTAG 2100

GGTCTCCATC CCTAATTAAG GGATAACTAG TACTAACAAC TAGCACAAAC TATCTAGATT 2160

TCAGATATCA CCACATTGAC AAACAATCAT CCATGAACTA TGATCCATTC ATCCATCCAT 2220

CAAAAAATAA ATCGGTATTT CGAGAACGAT AGAAGAAATG AAGTCGGCTC ACCTTTCTTG 2280

GTCACCATTT GAGTTTGTTG GTGCCTGAGA ATCCATGGTC GTCATCGTCG TCCTAGGGAT 2340

CGGCGGTGCT CCTCGTTGTT GGTAAAGTCG CCAGTGTGTA GTGCTAGCGC AACTGTCCAG 2400

GCGTGCAACG GTTGGCCGGC TGGAAAGGGC ATAGCGTATG GCTGGTTATT TTTAGGGTTT 2460

TGTTTTTTTA CTAATCTGCT AGTTGCCTTG CCATGTTGTC TTATTGGGCT AGGATCTAGG 2520

GCTTGTTACG CTGCTGTGTT GGGCTTGGTG TCCGGTTCAG CCTCAACTCA TTCATACAAA 2580

TCAGATTCAT ACAAAACAGG TATACACGTA TGAAATATCC ATGGATAATC AGGTTCGAAT 2640

TATTGTCCCC TAAACCCATA CACGTTTACC CAATGGATGG ATATTTTGTC TCATATCCAT 2700

ACACATGAGA CGATTTTTGT CCCATACCTG TGCTCTAATA GGAGAATTTC TCTCGGGATA 2760

GCGAGTATCG GATCCTCTAG AGTC 2784

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: oligonucleotide Zm13Oli2

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..24
(D) OTHER INFORMATION: /label= Zm13Oli2
/note= "oligonucleotide designated as Zm13Oli2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GTGGATTGAA CGGGACTGAG TTGG 24

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide Zm13Oli1

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..25
(D) OTHER INFORMATION: /label= Zm13Oli1
/note= "oligonucleotide designated as Zm13Oli1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

AAGTCTCCAA GACTTTGGTT ATTCC 25

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oligonucleotide Zm13Oli5

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..31
(D) OTHER INFORMATION: /label= Zm13Oli5

/note= "oligonucleotide designated as Zm1301i5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GGATCCATGG TTGCCGCCGG GTGAATGTAC G 31

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide BXOL2

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..24
(D) OTHER INFORMATION: /label= BXOL2
/note= "oligonucleotide designated as BXOL2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ACGGAAAACC TGAAGCACAC TCTC 24

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide TA29SBXOL2

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..49
(D) OTHER INFORMATION: /label= TA29SBXOL2
/note= "oligonucleotide designated as TA29SBXOL2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GTTTTTACTT AAAGAAATTA GCTACCATGA AAAAAGCAGT CATTAACGG 49

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i
i) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide PTA29OL5

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..27
(D) OTHER INFORMATION: /label= PTA29OL5
/note= "oligonucleotide designated as PTA29OL5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TGGCCATAAC TGAAATCAGG GTGAGAC 27

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4808 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: EcoRI-HindIII fragment of plasmid pTS218

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (18..401)
(D) OTHER INFORMATION: /label= 3'nos
/note= "3' regulatory sequence containing the polyadenylation site derived from Agrobacterium T-DNA nopaline synthase gene"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (402..737)
(D) OTHER INFORMATION: /label= barnase
/note= "coding region of the barnase gene of Bacillus amyloliquefaciens"

(ix) FEATURE:

(A) NAME/KEY: -
(B) LOCATION: complement (738..1944)
(D) OTHER INFORMATION: /label= PZM13
/note= "promoter region of the Zm13 gene of Zea mays"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1945..2281)
(D) OTHER INFORMATION: /label= 3'nos

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (2282..2554)
(D) OTHER INFORMATION: /label= barstar
/note= "coding region of the barstar gene of Bacillus amyloliquefaciens"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (2555..3099)
(D) OTHER INFORMATION: /label= PTA29
/note= "promoter region of the TA29 gene of Nicotiana tabacum"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 3100..3932
(D) OTHER INFORMATION: /label= 35S3
/note= ""35S3" promoter sequence derived from cauliflower mosaic virus isolate CabbB-JI"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 3933..4484
(D) OTHER INFORMATION: /label= bar
/note= "coding region of the phosphinothricin acetyltransferase gene"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 4485..4763
(D) OTHER INFORMATION: /label= 3'nos

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 2333..2356
(D) OTHER INFORMATION: /label= BXOL2
/note= "region corresponding to oligonucleotide BXOL2"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (2538..2586)
(D) OTHER INFORMATION: /label= TA29SBXOL2
/note= "region complementary to oligonucleotide TA29SBXOL2"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (2800..2823)
(D) OTHER INFORMATION: /label= PTA29OL5

/note= "region complementary to part of oligonucleotide PTA29OL5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

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CGGTCTGAAT TTCTGAAGCC TGATGTATAG TTAATATCCG CTTCACGCCA TGTTCGTCCG 540

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CGCGCGGTGT CATCTATGTT ACTAGATCGG GAAGATCCTC TAGAGTCGAC CTGCAGGCAT 4800

GCAAGCTT 4808

FIGURE 1

1. Action : Transform corn embryos (e.g. H99) with male-sterility gene S, linked to herbicide resistance gene bar (Example 5)
   Result : transformed plants with genotype S/s

2. Action : Transform corn embryos (e.g. H99) with fertility-restorer gene R (Example 6)
   Result : transformed plants with genotype R/r

3. Action : Transform corn embryos (e.g; H99) with maintainer gene P (Example 3)
   Result : transformed plants with genotype P/p

4. Action : Cross S/s,r/r x s/s,R/r.
   Select offspring for presence of both S and R genes by means of PCR.
   Result : plants with genotype S/s,R/r

5. Action : Self selected plants of 4 (optional)
   Result : Progeny plants with 9 different genotypes

gamete ♀ ♂→ ↓	S,R	S,r	s,R	s,r
S,R	S/S,R/R	S/S,R/r	S/s,R/R	S/s,R/r
S,r	S/S,R/r	S/S,r/r**	S/s,R/r	S/s,r/r**
s,R	S/s,R/R	S/s,R/r	s/s,R/R	s/s,R/r
s,r	S/s,R/r	S/s,r/r**	s/s,R/r	s/s,r/r

** male-sterile plants

6. Action : self male-fertile progeny plants of 5 (Optional)
   Result :

   6.1. Self of S/S,R/R : 100 % male-fertile plants
   Self of S/s,R/R : 100 % male-fertile plants
   Self of s/s,R/R : 100 % male-fertile plants
   Self of s/s,R/r : 100 % male-fertile plants
   Self of s/s,r/r : 100 % male-fertile plants

   6.2 Self of S/s,R/r : Same progeny as in 5
   13/16 male-fertile plants with 4/13 herbicide sensitive

FIGURE 1 (continued 1)

6.3 Self of S/S,R/r : Progeny as follows :

gamete ♀ ↓ ♂→	S,R	S,r
S,R	S/S,R/R	S/S,R/r
S,r	S/S,R/r	S/S,r/r**

** male-sterile plants

Thus : 3/4 male-fertile plants, 0% herbicide sensitive
All male-sterile plants are of genotype S/S,r/r

7. Action : Cross
♀ : P/p (from 3) x ♂ : S/s,R/r (from 4)
this equals in fact
♀ : s/s,r/r,P/p x ♂ : S/s,R/r,p/p
Result : Progeny with the following genotypes

gamete ♀ ↓ ♂→	S,R,p	S,r,p	s,R,p	s,r,p
s,r,P	S/s,R/r,P/p	S/s,r/r,P/p	s/s,R/r,P/p	s/s,r/r,P/p
s,r,p	S/s,R/r,p/p	S/s,r/r,p/p	s/s,R/r,p/p	s/s,r/r,p/p

8. Action : From offspring of 7, select plants with genotype S/s,r/r,P/p by screening, by means of PCR and/or Southern blotting, for presence of S and P gene and absence of R gene.
Result : plants with genotype S/s,P/p

FIGURE 1 (continued 2)

9. Action : Self plants with genotype S/s,P/p (from 8)
Result : progeny with the following genotypes

gamete ♀ ↓ ♂→	S,P	S,p	s,P	s,p
S,P	S/S,P/P	S/S,P/p	S/s,P/P	S/s,P/p
S,p	S/S,P/p	S/S,p/p**	S/s,P/p	S/s,p/p**
s,P	S/s,P/P	S/s,P/p	s/s,P/P	s/s,P/p
s,p	S/s,P/p	S/s,p/p**	s/s,P/p	s/s,p/p

** male-sterile plants
Shaded genotypes cannot develop because male gametes (pollen) are killed off by expression of the maintainer gene P.

10. Action : self male fertile plants of 9.
Result
10.1. Self of s/s,P/p : 100 % male-fertile plants
Self of s/s,p/p : 100 % male-fertile plants

10.2 Self of S/s,P/p : Same progeny as in 9
5/8 male-fertile plants with
2/5 herbicide sensitive

10.3 Self of S/S,P/p : Progeny as follows :

gamete ♀ ↓ ♂→	S,P	S,p
S,P	S/S,P/P	S/S,P/p
S,p	S/S,P/p	S/S,p/p**

** male-sterile plants
Shaded genotypes cannot develop because male gametes (pollen) are killed off by expression of the maintainer gene P.

FINAL RESULT

- 1/2 male-fertile plants, 0% herbicide sensitive. All these plants are maintainer plants
- 1/2 male sterile plants. All homozygous for the male-sterility gene S.

CLAIMS

1. A cell of a maintainer plant, the nuclear genome of which contains: 1) at a first locus, a male-sterility genotype in homozygous condition; and 2) at a second locus, a maintainer gene in heterozygous condition; said first and second loci preferably being unlinked; said maintainer gene being a foreign DNA sequence, preferably a foreign chimaeric DNA sequence, including:

a) a fertility-restorer gene that comprises :

i) a fertility-restorer DNA encoding a restorer RNA and/or protein or polypeptide which, when produced or overproduced in cells, preferably stamen cells, of said plant, prevents phenotypic expression of said nuclear male-sterility genotype which would render said plant male-sterile in the absence of said restorer RNA, protein or polypeptide in said stamen cells and

ii) a restorer promoter capable of directing expression of said fertility-restorer DNA at least in said cells, preferably said stamen cells, so that said phenotypic expression of said nuclear male-sterility genotype is prevented, said fertility-restorer DNA being in the same transcriptional unit as, and under the control of, said restorer promoter and

b) a pollen-lethality gene that is selectively expressed in microspores and/or pollen of said plant to prevent the production of functional pollen and that comprises :

iii) a pollen-lethality DNA coding for a pollen-lethality RNA and/or protein or polypetide that, when produced or overproduced in said microspores and/or pollen, significantly disrupts the metabolism, functioning and/or

development of said microspores and/or pollen and

iv) a pollen-specific promoter capable of directing expression of said pollen-lethality DNA selectively in said microspores and/or pollen said pollen-lethality DNA being in the same transcriptional unit as, and under the control of, said pollen-specific promoter.

2. The cell of claim 1, in which said maintainer gene also contains, preferably in said second locus, a first marker gene which comprises :

v) a first marker DNA encoding a first marker RNA and/or protein or polypeptide which, when present at least in a first specific tissue or specific cells of said plant, renders said plant easily separable from other plants which do not contain said first marker RNA, protein or polypeptide at least in said first specific tissue or specific cells and

vi) a first marker promoter capable of directing expression of said first marker DNA at least in said first specific tissue or specific cells, said first marker DNA being in the same transcriptional unit as, and under the control of, said first marker promoter.

3. The cell of claim 1 or 2, in which said male-sterility genotype is foreign to said plant and is a male-sterility gene that is a foreign DNA sequence, preferably a foreign chimaeric DNA sequence, comprising:

1) a male-sterility DNA encoding a sterility RNA and/or protein or polypeptide which, when produced or overproduced in said stamen cells of said plant in the absence of said restorer RNA, protein or polypeptide, significantly disturbs the metabolism,

functioning and/or development of said stamen cells and

2) a sterility promoter capable of directing expression of said male-sterility DNA selectively in said stamen cells, said male-sterility DNA being in the same transcriptional unit as, and under the control of, said sterility promoter.

4. The cell of claim 3 in which said male-sterility genotype also contains, preferably in said first locus, a second marker gene which comprises:

3) a second marker DNA encoding a second marker RNA and/or protein or polypeptide which, when present at least in a second specific tissue or specific cells of said plant, renders said plant easily separable from other plants which do not contain said second marker RNA, protein or polypeptide at least in said second specific tissue or specific cells and

4) a second marker promoter capable of directing expression of said second marker DNA at least in said specific tissue or specific cells, said second marker DNA being in the same transcriptional unit as, and under the control of, said second marker promoter.

5. The cell of claim 3 or 4, in which said male-sterility DNA encodes Barnase.

6. The cell of any one of claims 3 to 5, in which said sterility promoter is selected among the TA29 promoter of Nicotiana tabacum and the promoters of SEQ ID nos. 7 to 10.

7. The cell of claim 5 or 6, in which said restorer DNA encodes Barstar.

8. The cell of any one of claims 1 to 7, in which said restorer promoter is selected among the TA29 promoter of Nicotiana tabacum and the promoters of SEQ ID nos. 7 to 10.

9. The cell of claim 1 or 2, in which said male-sterility genotype is endogenous to said plant and is homozygous for a recessive allele.

10. The cell of claim 9, in which said fertility-restorer gene is the dominant allele of the endogenous male-sterility genotype, preferably under the control of its natural promoter.

11. The cell of any one of claims 1 to 10, in which said pollen-lethality DNA encodes a ribonuclease, preferably RNAse T1 or Barnase.

12. The cell of any one of claims 1 to 11, in which said pollen-specific promoter is the Zm13 promoter of SEQ ID no. 1.

13. The cell of any one of claims 2 to 12, in which said first marker DNA or said second marker DNA is: an herbicide resistance gene, particularly an sfr or sfrv gene; a gene encoding a modified target enzyme for an herbicide having lower affinity for the herbicide, particularly a modified 5-enolpyruvylshikimate-3 phosphate synthase as a target for glyphosate or a modified glutamine synthetase as a target for a glutamine synthetase inhibitor such as phosphinotricine; a gene encoding a protein which confers resistance to the herbicide sulfonylurea; a gene encoding a protein which confers resistance to the herbicide, bromoxynil; a gene encoding a protein which confers resistance to the herbicide 2,4 D; a gene encoding a protein or a polypeptide conferring a color to at least said first or second specific tissue or specific cells, particularly the A1 gene or the glucuronidase gene; a gene encoding a protein or a polypeptide conferring a stress tolerance to said plant, particularly a gene encoding Mn-superoxide dismutase; a gene encoding a protein or a polypeptide conferring a disease or pest resistance, particularly

a gene encoding a Bacillus thuringiensis endotoxin that confers insect resistance; or a gene encoding a bactericidal peptide that confers a bacterial resistance.

14. The cell of any one of claims 2 to 13, in which said first marker promoter or said second marker promoter is: a constitutive promoter, particularly a 35S promoter, a 35S'3 promoter, a PNOS promoter or a POCS promoter; a wound-inducible promoter, particularly a TR1' or TR2' promoter; a promoter which directs gene expression selectively in plant tissue having photosynthetic activity, particularly an SSU promoter; or a promoter which directs gene expression selectively in leaf cells, petal cells or seed cells, particularly seed coat cells.

15. The cell of any one of claims 2 to 12, wherein said first marker DNA and said second marker DNA are different.

16. A cell of a plant, the nuclear genome of which contains the maintainer gene of any one of claims 1 to 14.

17. A plant cell culture consisting essentially of the plant cells of any one of claims 1 to 16.

18. A plant, particularly corn, oilseed rape, wheat, rice, sunflower, sugarbeet, tomato, lettuce, peppers, sorghum, soybean, pea, alfalfa, grasses, clovers, carrot, cabbages, leek, onion, tobacco, petunia, cacao and citrus, more particularly corn, oilseed rape, wheat and rice, consisting essentially of the plant cells of any one of claims 1 to 16.

19. A seed of the plant of claim 18.

20. The maintainer gene of any one of claims 1 to 14.

21. A vector for transforming a cell of a plant, comprising the maintainer gene of any one of claims 1 to 14, particularly pTS210.

22. A method to maintain a homogeneous population of male-sterile plants or their seed, the nuclear genome of which contain, at said first locus of any one of claims 1, 3-6 and 9, said male-sterility genotype of any one of claims 1, 3-6 and 9 in homozygous condition; said method comprising the step of crossing said male-sterile plants with the plant of claim 18.

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/82; C12N15/29; C12N15/55; C12N15/31
C12N5/10; A01H1/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	WO,A,9 008 828 (PALADIN HYBRIDS) 9 August 1990 see page 12, line 15 - page 15, line 6	1-22
A	EP,A,0 412 911 (PLANT GENETIC SYSTEMS) 13 February 1991 cited in the application see the whole document	1-22
A	NATURE vol. 357, 4 June 1992, LONDON GB pages 384 - 387 MARIANI, C., ET AL. 'A chimaeric ribonuclease-inhibitor gene restores fertility to male sterile plants' see the whole document	1-22

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
21 OCTOBER 1993	05 -11- 1993
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9301489
SA 76008

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 21/10/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		EP-A-	0456706	21-11-91
		JP-T-	4504355	06-08-92
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